



Defendant marks certain of its Cipro ®, Ciprodex ®, Fludara ®, Legend ®, and Proleukin ® branded products with the expired patents with the intent to deceive competitors and the public, and to gain a competitive advantage in the market.

3.

Plaintiff seeks an award of monetary damages against Defendant pursuant to 35 U.S.C. § 292 (b) of up to \$500 for each offense, with one-half going to the use of the United States and the other half going to the person bringing the action.

### **THE PARTIES**

4.

Plaintiff is a person residing in Lakeland, Tennessee.

5.

Defendant BAYER AKTIENGESCELLSCHAFT CORPORATION d/b/a Bayer AG is a Delaware corporation with its principal place of business in Pittsburgh, Pennsylvania. Defendant's registered agent for service of process is The Company Corporation, 2711 Centerville Road, Suite 400, Wilmington, DE 19808.

6.

Defendant BAYER CORPORATION is a Delaware corporation with its principal place of business in Pittsburgh, Pennsylvania. Defendant's registered agent for service of process is Corporation Service Company, 2908 Poston Avenue, Nashville, TN 37203.

7.

Defendant BAYER HEALTHCARE, LLC, is a Delaware corporation with its principal place of business in Tarrytown, New York. Defendant's registered agent for service of process is Corporation Service Company, 2908 Poston Avenue, Nashville, TN 37203.

8.

Defendant BAYER HEALTHCARE PHARMACEUTICALS, INC., is a Delaware corporation with its principal place of business in Wayne, New Jersey. Defendant's registered agent for service of process is Corporation Service Company, 2908 Poston Avenue, Nashville, TN 37203.

### **JURISDICTION AND VENUE**

9.

This Court has jurisdiction over this action pursuant to 28 U.S.C. §§ 1331 and 1338(a).

10.

Venue is proper in this District under 28 U.S.C. §§ 1391(c) and 1395(a) because, at least in part, Defendant Bayer's products that are the subject-matter of this Complaint, were and are advertised, offered for sale, and sold within this District.

11.

This Court has personal jurisdiction over Defendant Bayer because Defendant has sold and continues to sell falsely marked products in Tennessee and in this District, and/or in the streams of commerce with knowledge that said products would be sold in Tennessee and in this District. Upon information and belief, such sales by Defendant are substantial, continuous, and systematic.

12.

Plaintiff brings this action under 35 U.S.C. § 292(b), which provides that any person may sue for civil money penalties for false patent marking.

**GENERAL ALLEGATIONS**

13.

Defendant Bayer has in the past manufactured and marketed (or caused to be manufactured and marketed), and presently manufactures and markets (or causes to be manufactured and marketed), products for sale to the general consuming public, including Baytril ®, Cipro ®, Ciprodex ®, Fludara ®, Legend ®, and Proleukin ®.

14.

Specifically, Defendant Bayer has and continues to market the following: Baytril ®, a veterinary product, is a fluoroquinolone antibiotic used to treat many certain kinds of bacterial infections in dogs and cats; Cipro ® is a fluoroquinolone antibiotic used to treat certain kinds of bacterial infections in humans; Ciprodex ® is an ear drop containing ciprofloxacin and dexamethasone, used to treat middle ear infections typically contracting by children; Fludara ® is a chemotherapy drug, commonly used to treat chronic lymphocytic leukemia; Legend ®, a veterinary product, is a hyaluronate sodium product designed to treat acute joint damage in horses; and Proleukin ® is a chemotherapy drug, usually used for advanced renal cell cancer or advanced melanoma.

15.

Baytril ®, including its packaging and/or labeling, have been and continue to be marked with United States patent number 4,670,444 (hereinafter referred to as the “ ‘444 Patent”).

16.

The ‘444 Patent has expired, but Defendant Bayer nevertheless continues using the improper patent marking on Baytril ®, with the intent to deceive the public and to gain competitive advantage in the market.



17.

Cipro ® and/or its packaging have been and continue to be marked with the ‘444 patent referenced in paragraphs 15 and 16, above, as well as United States patent number 4,844,902 (hereinafter referred to as the “ ‘902 Patent”).

18.

The ‘444 Patent and ‘902 Patent have expired, but Defendant Bayer nevertheless continues using the improper patent markings on Cipro ®, with the intent to deceive the public and to gain competitive advantage in the market.

19.

In addition, Ciprodex ® and/or its packaging have been and continue to be marked with the ‘902 patent referenced in paragraphs 17 and 18, above.

20.

Again, although the ‘902 Patent has expired, Defendant Bayer nevertheless continues using the improper patent marking on Ciprodex ®, with the intent to deceive the public and to gain competitive advantage in the market.

21.

Fludara ® and/or its packaging have been and continue to be marked with United States patent number 4,357,324 (hereinafter referred to as the “ ‘324 Patent”).

22.

The ‘324 Patent has expired, but Defendant Bayer nevertheless continues using the improper patent marking on Fludara ®, with the intent to deceive the public and to gain competitive advantage in the market.

23.

Legend ® and/or its packaging have been and continue to be marked with United States patent number 4,808,576 (hereinafter referred to as the “ ‘576 Patent”).

24.

The ‘576 Patent has expired, but Defendant Bayer nevertheless continues using the improper patent marking on Legend ®, with the intent to deceive the public and to gain competitive advantage in the market.

25.

Proleukin ® and/or its packaging have been and continue to be marked with the following United States patent numbers: 4,530,787; 4,569,790; 4,604,377; 4,748,234; 4,572,798; 4,853,332; and 4,959,314 (hereinafter referred to as the “ ‘787 Patent,” the “ ‘790 Patent,” the “ ‘377 Patent,” the “ ‘234 Patent,” the “ ‘798 Patent,” the “ ‘332 Patent,” and the “ ‘314 Patent,” respectively).

26.

Although the ‘787 Patent, ‘790 Patent, ‘377 Patent, ‘234 Patent, ‘798 Patent, ‘332 Patent, and ‘314 Patent have all expired, Defendant Bayer nevertheless continues using the improper patent markings on Proleukin ®, with the intent to deceive the public and to gain competitive advantage in the market.

27.

When a patent expires, all monopoly rights to the patent terminate irrevocably. Therefore, a product marked with an expired patent is not currently patented by such expired patent. In other words, the product is unpatented.

28.

Marking products with expired patents is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products, which, in turn, causes harm to the consuming public, including Plaintiff, by quelling product innovation and price competition.

29.

Defendant Bayer is a sophisticated company with many decades of experience applying for and obtaining patents, and therefore knows that patents do not have an indefinite duration but, rather, expire.

30.

Upon information and belief, Defendant Bayer employs an in-house legal department.

31.

Upon information and belief, attorneys in Defendant Bayer's in-house legal department are responsible for Defendant's intellectual property and marketing, labeling, and advertising law.

33.

Defendant Bayer by itself or by its representatives cannot genuinely believe that a patent does not expire, and that prospective patent rights apply even after its expiration.

34.

Defendant Bayer knew that both the '444 Patent, marked on Baytril ® as identified herein, had expired. Also, Defendant knew that the same '444 Patent, as well as the '902 Patent, both marked on Cipro ® as identified herein, had expired. Furthermore, Defendant knew that the same '902 Patent was expired when it was marked on Ciprodex ®, as identified herein.

Similarly, Defendant knew that '324 Patent, marked on Fludara ® as identified herein, had expired. Additionally, Defendant knew that the '576 Patent, marked on Legend ® as identified herein, had expired. Finally, Defendant knew that the '787 Patent, '790 Patent, '377 Patent, '234 Patent, '798 Patent, '332 Patent, and '314 Patent, each marked on Proleukin ® as identified herein, had all expired.

35.

After the '444 Patent expired, Defendant Bayer marked, or caused to be marked, said expired patent number upon Baytril ®, including its packaging and/or labeling. Also, after the '444 Patent and '902 Patent expired, Defendant marked, or caused to be marked, said expired patent numbers upon Cipro ®, including its packaging and/or labeling. Similarly, after the '902 Patent expired, Defendant marked, or caused to be marked, said expired patent number upon Ciprodex ®, including its packaging and/or labeling. Additionally, after the '324 Patent expired, Defendant marked, or caused to be marked, said expired patent number upon Fludara ®, including its packaging and/or labeling. Furthermore, after the '576 Patent expired, Bayer marked, or caused to be marked, said expired patent number upon Legend ®, including its packaging and/or labeling. Finally, after the expiration of the '787 Patent, '790 Patent, '377 Patent, '234 Patent, '798 Patent, '332 Patent, and '314 Patent, Bayer marked, or caused to be marked, said expired patent numbers upon Proleukin ®, including its packaging and/or labeling.

36.

Defendant Bayer knew that the patents marked on the products identified herein were expired during time periods Defendant was marking products with such expired patents.

37.

Because all monopoly rights in the aforementioned expired patents have terminated, Defendant Bayer cannot have any reasonable belief that Baytril ®, Cipro ®, Ciprodex ®, Fludara ®, Legend ®, and Proleukin ® are patented or otherwise covered by the expired patents marked upon the packaging of these products.

38.

By repeatedly marking Baytril ®, Cipro ®, Ciprodex ®, Fludara ®, Legend ®, and Proleukin ® with expired patents, Defendant Bayer has committed numerous violations of 35 U.S.C. § 292(a).

39.

Defendant Bayer has committed such violations of 35 U.S.C. § 292(a) with an intent to deceive competitors and the public.

40.

Plaintiff seeks an award of monetary damages against Defendant Bayer, one half of which shall be paid to the United States pursuant to 35 U.S.C. § 292(b).

**COUNT ONE: THE '444 PATENT (BAYTRIL ®)**

41.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

42.

United States Patent Number 4,670,444 was filed on May 29, 1984, and issued on June 2, 1987. (Please see United States Patent No. 4,670,444, attached hereto as Exhibit 'A').

43.

The '444 Patent expired on December 9, 2007 (following receipt of a three-year extension). (Please see "Certificate Extending Patent Term," attached hereto as Exhibit 'B').

44.

Defendant Bayer marketed for sale to the public the product known as Baytril ®, marked with the '444 Patent.

45.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Baytril ® with the '444 Patent, and any and all other products marked with the '444 Patent, subsequent to the date the patent expired with the intent to deceive the public.

46.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '444 Patent had expired.

47.

Defendant Bayer cannot genuinely believe that the '444 Patent applies even after it expired.

48.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law.

As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

49.

Defendant Bayer has falsely marked Baytril ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

50.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

51.

Defendant Bayer's false marking of products with the '444 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

52.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;

- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking “offense” (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney’s fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT TWO: THE ‘444 PATENT (CIPRO ®)**

53.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

54.

United States Patent Number 4,670,444 was filed on May 29, 1984, and issued on June 2, 1987. (Please see United States Patent No. 4,670,444, attached hereto as Exhibit ‘A’).

55.

The ‘444 Patent expired on December 9, 2007 (following receipt of a three-year extension). (Please see “Certificate Extending Patent Term,” attached hereto as Exhibit ‘B’).

56.

Defendant Bayer marketed for sale to the public the product known as Cipro ®, marked with the ‘444 Patent.



57.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Cipro ® with the ‘444 Patent, and any and all other products marked with the ‘444 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Cipro ® Labels (Cipro® HC at p. 4 of 5, Cipro® HC OTIC at p. 4 of 5), attached hereto as Exhibit ‘C’).

58.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the ‘444 Patent had expired.

59.

Defendant Bayer cannot genuinely believe that the ‘444 Patent applies even after it expired.

60.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant’s intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

61.

Defendant Bayer has falsely marked Cipro ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

62.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

63.

Defendant Bayer's false marking of products with the '444 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

64.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;

- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT THREE: THE ‘902 PATENT (CIPRO ®)**

65.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

66.

United States Patent Number 4,844,902 was filed on February 11, 1988, and issued on July 4, 1989. (Please see United States Patent No. 4,844,902, attached hereto as Exhibit ‘D’).

67.

The ‘902 Patent expired on February 11, 2008.

68.

Defendant Bayer marketed for sale to the public the product known as Cipro ®, marked with the ‘902 Patent.

69.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Cipro ® with the ‘902 Patent, and any and all other products marked with the ‘902 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Cipro ® Labels (Cipro® HC at p. 4 of 5, Cipro® HC OTIC at p. 4 of 5), attached hereto as Exhibit ‘C’).

70.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '902 Patent had expired.

71.

Defendant Bayer cannot genuinely believe that the '902 Patent applies even after it expired.

72.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

73.

Defendant Bayer has falsely marked Cipro ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

74.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

75.

Defendant Bayer's false marking of products with the '902 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

76.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT FOUR: THE ‘902 PATENT (CIPRODEX ®)**

77.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

78.

United States Patent Number 4,844,902 was filed on February 11, 1988, and issued on July 4, 1989. (Please see United States Patent No. 4,844,902, attached hereto as Exhibit ‘D’).

79.

The ‘902 Patent expired on February 11, 2008.

80.

Defendant Bayer marketed for sale to the public the product known as Ciprodex ®, marked with the ‘902 Patent.

81.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Ciprodex ® with the ‘902 Patent, and any and all other products marked with the ‘902 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Ciprodex ® label, attached hereto as Exhibit ‘E’).

82.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the ‘902 Patent had expired.

83.

Defendant Bayer cannot genuinely believe that the ‘902 Patent applies even after it expired.

84.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

85.

Defendant Bayer has falsely marked Ciprodex ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

86.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

87.

Defendant Bayer's false marking of products with the '902 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

88.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking “offense” (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney’s fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT FIVE: THE ‘324 PATENT (FLUDARA ®)**

89.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

90.

United States Patent Number 4,357,324 was filed on February 24, 1981, and issued on November 2, 1982. (Please see United States Patent No. 4,357,324, attached hereto as Exhibit ‘F’).

91.

The ‘324 Patent expired on February 24, 2003 (following receipt of a two-year extension).



92.

Defendant Bayer marketed for sale to the public the product known as Fludara ®, marked with the '324 Patent.

93.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Fludara ® with the '324 Patent, and any and all other products marked with the '324 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Fludara Label ® (p.10 of 11), attached hereto as Exhibit 'G').

94.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '324 Patent had expired.

95.

Defendant Bayer cannot genuinely believe that the '324 Patent applies even after it expired.

96.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters,

including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

97.

Defendant Bayer has falsely marked Fludara ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

98.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

99.

Defendant Bayer's false marking of products with the '324 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

100.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;

- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT SIX: THE '576 PATENT (LEGEND ®)**

101.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

102.

United States Patent Number 4,808,576 was filed on April 28, 1986, and issued on February 29, 1989. (Please see United States Patent No. 4,808,576, attached hereto as Exhibit 'H').

103.

The '576 Patent expired on April 28, 2006.

104.

Defendant Bayer marketed for sale to the public the product known as Legend®, marked with the '576 Patent.

105.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Legend ® with the '576 Patent, and any and all other products marked with the '576 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Legend® Label, attached hereto as Exhibit 'I').

106.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '576 Patent had expired.

107.

Defendant Bayer cannot genuinely believe that the '576 Patent applies even after it expired.

108.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

109.

Defendant Bayer has falsely marked Legend ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

110.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

111.

Defendant Bayer's false marking of products with the '576 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

112.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT SEVEN: THE '787 PATENT (PROLEUKIN ®)**

113.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

114.

United States Patent Number 4,530,787 was filed on October 17, 1984, and issued on July 23, 1985. (Please see United States Patent No. 4,530,787, attached hereto as Exhibit 'J').

115.

The '787 Patent expired on March 17, 2004.

116.

Defendant Bayer marketed for sale to the public the product known as Proleukin ®, marked with the '787 Patent.

117.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Proleukin ® with the '787 Patent, and any and all other products marked with the '787 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Proleukin Label, attached hereto as Exhibit 'K').

118.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '787 Patent had expired.

119.

Defendant Bayer cannot genuinely believe that the '787 Patent applies even after it expired.

120.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

121.

Defendant Bayer has falsely marked Proleukin ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

122.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

123.

Defendant Bayer's false marking of products with the '787 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

124.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking “offense” (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney’s fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT EIGHT: THE ‘790 PATENT (PROLEUKIN ®)**

125.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

126.

United States Patent Number 4,569,790 was filed on March 28, 1984, and issued on February 11, 1986. (Please see United States Patent No. 4,569,790, attached hereto as Exhibit ‘L’).

127.

The ‘787 Patent expired on March 28, 2004.



128.

Defendant Bayer marketed for sale to the public the product known as Proleukin ®, marked with the ‘790 Patent.

129.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Proleukin ® with the ‘790 Patent, and any and all other products marked with the ‘790 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Proleukin Label, attached hereto as Exhibit ‘K’).

130.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the ‘790 Patent had expired.

131.

Defendant Bayer cannot genuinely believe that the ‘790 Patent applies even after it expired.

132.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant’s intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters,

including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

133.

Defendant Bayer has falsely marked Proleukin ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

134.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

135.

Defendant Bayer's false marking of products with the '790 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

136.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;

- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT NINE: THE '377 PATENT (PROLEUKIN ®)**

137.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

138.

United States Patent Number 4,604,377 was filed on March 21, 1985, and issued on August 5, 1987. (Please see United States Patent No. 4,604,377, attached hereto as Exhibit 'M').

139.

The '377 Patent expired on March 21, 2004.

140.

Defendant Bayer marketed for sale to the public the product known as Proleukin ®, marked with the '377 Patent.

141.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Proleukin ® with the '377 Patent, and any and all other products marked with the '377 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Proleukin Label, attached hereto as Exhibit 'K').

142.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '377 Patent had expired.

143.

Defendant Bayer cannot genuinely believe that the '377 Patent applies even after it expired.

144.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

145.

Defendant Bayer has falsely marked Proleukin ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

146.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

147.

Defendant Bayer's false marking of products with the '377 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

148.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT TEN: THE '234 PATENT (PROLEUKIN ®)**

149.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

150.

United States Patent Number 4,748,234 was filed on March 25, 1986, and issued on May 31, 1988. (Please see United States Patent No. 4,748,234, attached hereto as Exhibit 'N').

151.

The '234 Patent expired on March 25, 2006.

152.

Defendant Bayer marketed for sale to the public the product known as Proleukin ®, marked with the '234 Patent.

153.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Proleukin ® with the '234 Patent, and any and all other products marked with the '234 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Proleukin Label, attached hereto as Exhibit 'K').

154.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '234 Patent had expired.

155.

Defendant Bayer cannot genuinely believe that the '234 Patent applies even after it expired.

156.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

157.

Defendant Bayer has falsely marked Proleukin ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

158.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

159.

Defendant Bayer's false marking of products with the '234 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

160.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking “offense” (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney’s fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT ELEVEN: THE ‘798 PATENT (PROLEUKIN ®)**

161.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

162.

United States Patent Number 4,572,798 was filed on December 6, 1984, and issued on February 25, 1986. (Please see United States Patent Number 4, 572,798, attached hereto as Exhibit ‘O’).

163.

The ‘798 Patent expired on December 6, 2004.



164.

Defendant Bayer marketed for sale to the public the product known as Proleukin ®, marked with the '798 Patent.

165.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Proleukin ® with the '798 Patent, and any and all other products marked with the '798 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Proleukin Label, attached hereto as Exhibit 'K').

166.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '798 Patent had expired.

167.

Defendant Bayer cannot genuinely believe that the '798 Patent applies even after it expired.

168.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters,

including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

169.

Defendant Bayer has falsely marked Proleukin ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

170.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

171.

Defendant Bayer's false marking of products with the '798 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

172.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;

- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT TWELVE: THE '332 PATENT (PROLEUKIN ®)**

173.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

174.

United States Patent Number 4,853,332 was filed on December 21, 1984, and issued on August 1, 1989. (Please see United States Patent Number 4,853,332, attached hereto as Exhibit 'P').

175.

The '332 Patent expired on August 1, 2006.

176.

Defendant Bayer marketed for sale to the public the product known as Proleukin ®, marked with the '332 Patent.

177.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Proleukin ® with the '332 Patent, and any and all other products marked with the '332 Patent, subsequent to the date the patent expired

with the intent to deceive the public. (Please see Proleukin Label, attached hereto as Exhibit ‘K’).

178.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the ‘332 Patent had expired.

179.

Defendant Bayer cannot genuinely believe that the ‘332 Patent applies even after it expired.

180.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant’s intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

181.

Defendant Bayer has falsely marked Proleukin ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

182.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

183.

Defendant Bayer's false marking of products with the '332 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

184.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking "offense" (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney's fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**COUNT THIRTEEN: THE '314 PATENT (PROLEUKIN ®)**

185.

Plaintiff restates and incorporates the foregoing paragraphs as if fully set forth herein.

186.

United States Patent Number 4,959,314 was filed on February 7, 1985, and issued on September 25, 1990. (Please see United States Patent No. 4,959,314, attached hereto as Exhibit 'Q').

187.

The '314 Patent expired on September 25, 2007.

188.

Defendant Bayer marketed for sale to the public the product known as Proleukin ®, marked with the '314 Patent.

189.

Defendant Bayer violated 35 U.S.C. § 292(a) by marking, or causing to be marked, the packaging, labeling, and/or product commonly known as Proleukin ® with the '314 Patent, and any and all other products marked with the '314 Patent, subsequent to the date the patent expired with the intent to deceive the public. (Please see Proleukin Label, attached hereto as Exhibit 'K').

190.

Upon information and belief, Defendant Bayer knew, on or about the date of expiration, that the '314 Patent had expired.

191.

Defendant Bayer cannot genuinely believe that the '314 Patent applies even after it expired.

192.

Upon information and belief, Defendant Bayer is a sophisticated company and has many decades of experience applying for, obtaining, and litigating patents. Defendant has committed a substantial amount of its resources to patent procurement and enforcement. Upon information and belief, Defendant has an in-house legal department and attorneys working therein are responsible for Defendant's intellectual property and marketing, labeling, and advertising law. As a sophisticated company, with in-house legal counsel that regularly handles patent matters, including but not limited to patent procurement and patent-related litigation, Defendant is aware of the requirements of 35 U.S.C. § 292.

193.

Defendant Bayer has falsely marked Proleukin ® as described with the intent to deceive the public, in violation of 35 U.S.C. § 292(a).

194.

Each false marking on the product(s) identified is likely to, or at least has the potential to, discourage or deter persons and companies from commercializing competing products.

195.

Defendant Bayer's false marking of products with the '314 Patent after it expired has wrongfully quelled competition with respect to such products, thereby causing harm to Plaintiff, the United States, and the general public.

196.

Defendant Bayer wrongfully and illegally advertised a patent monopoly which it did not possess and, as a result, has benefitted commercially and financially by maintaining false statements of patent rights.

WHEREFORE, Plaintiff demands a trial by jury and requests that the Court enter judgment as follows:

- (a) Enter judgment against Defendant and in favor of Plaintiff for the violations alleged in this Complaint;
- (b) Order Defendant to pay a civil monetary fine of up to five hundred dollars (\$500) per false marking “offense” (or falsely marked article), or an alternative amount as determined by the Court, one-half of which shall be paid to the United States;
- (c) Order Defendant to pay discretionary costs and prejudgment interest;
- (d) Award attorney’s fees to plaintiff pursuant to 35 USC § 285;
- (e) Order an accounting for any falsely marked products not presented at trial and an award by the Court of additional damages for any such falsely marked products; and
- (f) Grant Plaintiff such other and further relief, at law or in equity, to which Plaintiff is justly entitled.

**DEMAND FOR JURY TRIAL**

Pursuant to Fed. R. Civ. P. 38(b), Plaintiff demands a trial by jury on all issues so triable.

Respectfully submitted this 13th day of April, 2010.

/s/ W. Daniel Miles, III  
W. DANIEL “DEE” MILES, III \*

/s/ Roman A. Shaul  
ROMAN A. SHAUL (TN BPR # 024265)

/s/ Archie Grubb II  
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/s/ Kirk Caraway  
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EXHIBIT A  
PATENT 4,670,444

**United States Patent** [19]

Grohe et al.

[11] **Patent Number:** 4,670,444[45] **Date of Patent:** Jun. 2, 1987

[54] **7-AMINO-1-CYCLOPROPYL-4-OXO-1, 4-DIHYDRO-QUINOLINE-AND NAPHTHYRIDINE-3-CARBOXYLIC ACIDS AND ANTIBACTERIAL AGENTS CONTAINING THESE COMPOUNDS**

[75] **Inventors:** Klaus Grohe, Odenthal; Hans-Joachim Zeller, Velbert; Karl G. Metzger, Wuppertal, all of Fed. Rep. of Germany

[73] **Assignee:** Bayer Aktiengesellschaft, Leverkusen, Fed. Rep. of Germany

[21] **Appl. No.:** 614,923

[22] **Filed:** May 29, 1984

**Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 292,560, Aug. 13, 1981, abandoned, and a continuation-in-part of Ser. No. 436,112, Oct. 22, 1982, abandoned.

[30] **Foreign Application Priority Data**

Sep. 3, 1980 [DE] Fed. Rep. of Germany ..... 3033157  
Oct. 29, 1981 [DE] Fed. Rep. of Germany ..... 3142854

[51] **Int. Cl.<sup>4</sup>** ..... A61K 31/495; C07D 521/00

[52] **U.S. Cl.** ..... 514/300; 514/236; 514/254; 514/312; 544/127; 544/128; 544/295; 544/362; 544/363; 546/123; 546/156

[58] **Field of Search** ..... 544/362, 363, 295, 127, 544/128; 424/250; 546/156, 123; 514/254, 312, 300, 236

[56] **References Cited****U.S. PATENT DOCUMENTS**

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4,146,719 3/1979 Irikura ..... 544/363  
4,284,629 8/1981 Grohe et al. .... 544/279  
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4,522,819 6/1985 Fox, Jr. et al. .... 544/363  
4,556,709 12/1985 Grohe et al. .... 544/279

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2939786 4/1980 Fed. Rep. of Germany ..... 544/363

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Arzneimittelchemie I pp. 32-33, Georg Thieme Verlag Stuttgart, 1976.

*Primary Examiner*—Glennon H. Hollrah

*Assistant Examiner*—James H. Turnipseed

*Attorney, Agent, or Firm*—Sprung Horn Kramer & Woods

[57] **ABSTRACT**

The invention relates to 7-amino-1-cyclo-propyl-4-oxo-1, 4-dihydro-naphthyridine (or quinoline)-3-carboxylic acids of Formula I as defined in the specification. Also included in the invention is a process for the preparation of said compounds of Formula I and Ia. Further, the invention includes compositions containing the compounds of Formula I or Ia and the use of said compounds and compositions as antibacterial agents.

**22 Claims, No Drawings**

4,670,444

1

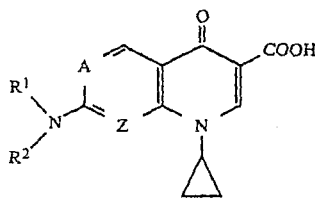
**7-AMINO-1-CYCLOPROPYL-4-OXO-1,4-DIHYDRO-QUINOLINE-AND NAPHTHYRIDINE-3-CARBOXYLIC ACIDS AND ANTIBACTERIAL AGENTS CONTAINING THESE COMPOUNDS**

This application is a continuation-in-part of our application Ser. No. 292,560 filed Aug. 13, 1981, now abandoned and a continuation-in-part of our application Ser. No. 436,112 filed Oct. 22, 1982 now abandoned.

The present invention relates to certain new 7-amino-1-cyclopropyl-4-oxo-1,4-dihydro-quinoline- and naphthyridine-3-carboxylic acid compounds, to processes for their production, to their use as antibacterial agents, and to feed additives containing these compounds.

It has already been disclosed that 7-amino-1-ethyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acids have antibacterial properties [see Eur. J. Med. Chem. 12, 541-547 (1977)]; and it has also been disclosed that 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acids possess antibacterial properties [J. Med. Chem. 23, 1358 (1980)].

According to the present invention there are provided compounds which are 7-amino-1-cyclopropyl-4-oxo-1,4-dihydro-quinoline- and naphthyridine-3-carboxylic acids of the formula



or a salt thereof,

in which A represents a nitrogen atom or CR<sup>3</sup>,

wherein R<sup>3</sup> denotes a hydrogen, a nitro group or a halogen atom (preferably a fluorine or chlorine atom), or a nitrile, carboxamide, carboxyl or ester group, and Z represents a nitrogen atom or C—H, and A and Z cannot simultaneously be nitrogen atoms, and R<sup>1</sup> and R<sup>2</sup> are identical or different and represent a hydrogen atom or a straight-chain or branched alkyl, alkenyl or alkynyl radical which has up to 12 (preferably up to 6) carbon atoms and is optionally substituted by radical(s) selected from hydroxyl, alkoxy, alkylmercapto or dialkylamino with 1 to 3 carbon atoms in each alkyl radical, nitrile, alkoxy carbonyl with 1 to 4 carbon atoms in the alcohol part, aryl and hetaryl, or furthermore represent a cycloalkyl radical with 3 to 6 carbon atoms, or, together with the nitrogen atom which they substitute and, if appropriate, a further hetero-atom (such as oxygen or sulphur, or NR<sup>4</sup>) form a 3-membered to 7-membered ring which can be monosubstituted disubstituted or polysubstituted by radical(s) selected from alkyl or alkenyl with up to 6 carbon atoms, hydroxyl, alkoxy or alkylmercapto with 1 to 3 carbon atoms, alkoxy carbonyl with 1 to 4 carbon atoms in the alcohol part, nitrile group and aryl, and which can furthermore possess a double bond, and R<sup>4</sup> represents a hydrogen atom, or a branched or straight-chain alkyl, alkenyl or alkynyl group which has up to 6 carbon atoms and is optionally

2

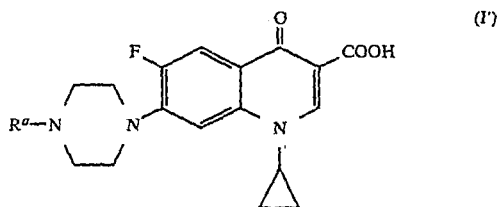
substituted by radical(s) selected from hydroxyl, alkoxy, alkylmercapto or dialkylamino with 1 to 3 carbon atoms per alkyl radical, and alkoxy carbonyl with 1 to 4 carbon atoms in the alcohol part, or represents an aralkyl group which is optionally substituted in the aryl radical by C<sub>1</sub>-C<sub>2</sub>-alkyl, halogen, preferably chlorine, NO<sub>2</sub> and/or NH<sub>2</sub> and has up to 4 (preferably 1 to 2) carbon atoms in the aliphatic part, or an optionally substituted phenyl or naphthyl group or a heterocyclic radical (such as a radical of pyridine, pyrimidine, thiazole or benzothiazole), or R<sup>4</sup> denotes an alkoxy carbonyl group which is optionally substituted by an aryl radical and has 1 to 4 carbon atoms in the alcohol part, an alkanoyl radical with 1 to 6 carbon atoms, an aryl radical, an optionally substituted C<sub>1</sub>-C<sub>3</sub>-alkyl- or aryl-(thio) carbonyl radical, an C<sub>1</sub>-C<sub>3</sub>-alkyl- or aryl-sulphonyl radical or an optionally substituted aminosulphonyl radical.

As used herein and unless otherwise specified, the term "aryl" is preferably mono- or bi-cyclic carbocyclic aryl, such as phenyl or naphthyl; the term "aralkyl" is preferably mono- or bi-cyclic carboxylic aryl-C<sub>1</sub>-C<sub>4</sub>-alkyl, such as benzyl, phenethyl, naphthyl-methyl and naphthyl-ethyl; the term "hetaryl" is preferably mono- or bi-cyclic, N-, O- or S-heteroaryl, such as pyridine, thiophene and furane; and the term "aroyl" is preferably benzoyl or naphthoyl.

The compounds of the present invention have a superior antibacterial action against both gram positive and gram negative bacteria, including *pseudomonas aeruginosa*, to that of the known quinolone- and azaquinolone-carboxylic acids.

The abovementioned aryl radicals, preferably the phenyl or naphthyl radical, are optionally monosubstituted di-substituted or polysubstituted by substituent(s) selected from halogen (preferably fluorine, chlorine and/or bromine), alkyl, alkoxy or alkylmercapto with 1 to 3 carbon atoms, aryloxy or arylmercapto, trifluoromethyl, nitro, nitrile and a carboxylic and ester group with 1 to 4 carbon atoms in the alcohol part.

Further according to the present invention and within the scope of the compounds identified above under Formula (I) there are now provided, as new compounds, 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperzino-quinoline-3-carboxylic acids of the general formula



or salts thereof,

in which, R<sup>a</sup> denotes a hydrogen atom or a methyl, ethyl or β-hydroxyethyl group.

Suitable salts are those of inorganic or organic acids, p.e. hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphonic acid, acetic acid, succinic acid, malic acid etc. Suitable salts are furthermore those of anorganic or organic bases, p.e. KOH, NaOH,

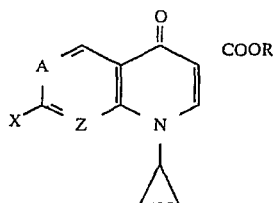
4,670,444

3  
Ca(OH)<sub>2</sub>, Al(OH)<sub>3</sub>, piperidine, morpholine, ethylamine, triethylamine etc.

The compounds of the formula (I') may contain various amounts of water.

According to the present invention, there is further provided a process for the production of a compound of the present invention characterized in that

(a) a quinolone-carboxylic acid of the formula



in which

R denotes a hydrogen atom,

A and Z have the abovementioned meaning and

X represents a halogen atom or an alkylsulphonyl group with 1 to 4 carbon atoms, is reacted with an amine of the formula

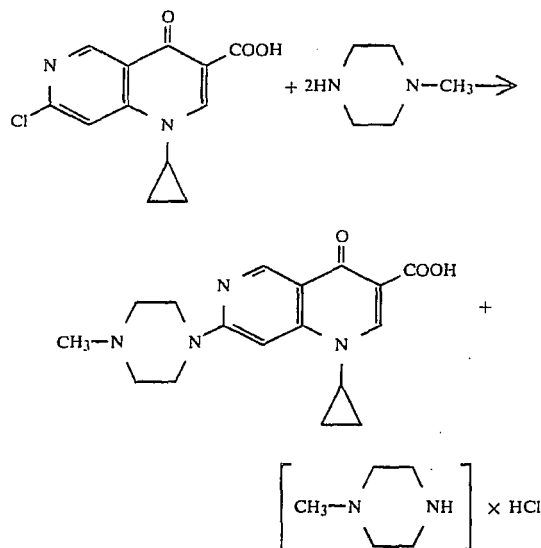


in which

R<sup>1</sup> and R<sup>2</sup> have the abovementioned meanings or

(b) a 7-halogeno-naphthyridine-3-carboxylic acid ester of a compound of formula (II), as given above, in which R denotes an alkyl radical and A, Z and X have the abovementioned meanings, is reacted with an amine of formula (II), as defined above, if appropriate in the presence of an acid-binding agent, (such as triethylamine or pyridine) and then the resulting 7-amino-naphthyridine-3-carboxylic acid ester is hydrolyzed under alkaline conditions.

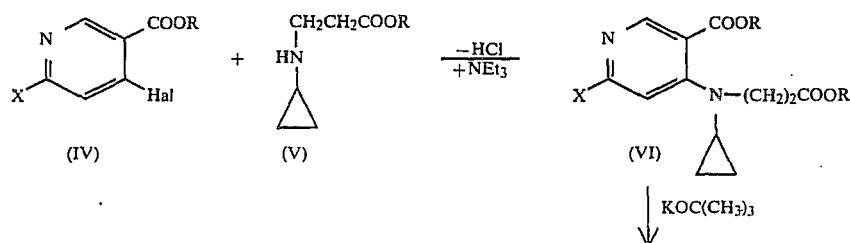
If, for example, 7-chloro-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid and N-methylpiperazine are used as reactants in the reaction, the course of the reaction variant (a) according to the present invention is illustrated by the following equation:



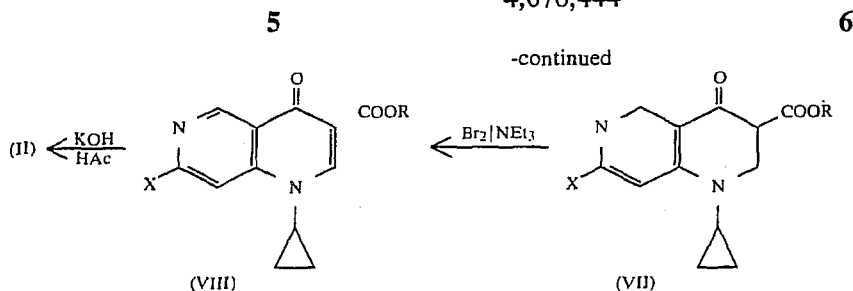
The starting compound of formula (II) can be prepared in the following manner (in which the formulae for the compounds concerned are given in the following reaction scheme):

The starting substance used is, for example, a 4-halogeno-pyridine-3-carboxylic acid ester of the formula (IV), which is substituted by a radical X in the 6-position, this ester is largely converted selectively into a monosubstitution product of the formula (IV), the halogen atom in the 4-position being replaced by the amine radical, with a  $\beta$ -cyclopropylamino-propionic acid ester of the formula (V), preferably a methyl or ethyl ester, which is readily accessible by reaction of corresponding acrylic acid ester with cyclopropylamine. The monosubstitution product of the formula (VI) is converted into a tetrahydro-naphthyridine-3-carboxylic acid ester of the formula (VII) by Dieckmann cyclisation in the presence of a strong base (such as potassium t-butyrate or sodium hydride). The carboxylic acid ester of the formula (VIII) is obtained from the ester of formula (VII) with bromine or sulphuryl chloride and triethylamine or pyridine as the dehydrohalogenating agent, and the compound of the formula (VIII) is saponified with an alkali to give the carboxylic acids of the formula (II) (in which R represents a hydrogen atom, A represents a nitrogen atom and Z represents CH).

One version of the abovementioned process for the production of a starting substance of formula (II) is represented by the reaction scheme:



4,670,444



Preferred possible diluents for the reaction variant (a) or (b) are ethanol, dioxane, toluene, dimethylformamide and dimethylsulphoxide.

Acid-binding agents which can be used in reaction 15 variant (b) are, preferably, alkali carbonates, alkali metal hydroxides or tert.-organic bases (such as, preferably, triethylamine and pyridine).

The reaction temperatures for reaction variants (a) or (b) can be varied within a substantial range. In general, 20 the reaction is carried out at a temperature between 20° and 180° C., preferably between 60° and 140° C.

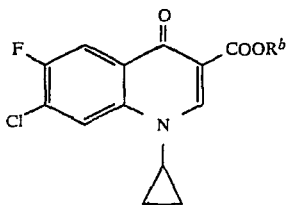
Both reaction variants can be carried out under normal pressure, but also under increased pressure, especially in the case of gaseous and low-boiling amines of the formula (III). In general, the reaction is carried out under pressures between 1 and 100 bars, preferably between 1 and 10 bars.

In carrying out reaction variant (a) or (b), 1 to 5 moles of amine, preferably 2 to 3 moles of amine, are employed per mole of carboxylic acid.

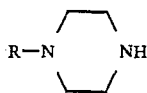
The 7-chloro-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid used as a starting material can be prepared in a multi-stage reaction sequence, for example starting from 4,6-dichloro-nicotinic acid ethyl ester, which is known (see Recueil Trav. chim. Pays-bas. 69, 687 (1950). The methyl ester is known from U.S. Pat. Nos. 4,066,645 and 4,075,210.

According to the present invention there is further provided a process for the production of a compound of the invention of Formula (I') in which

(a') 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid of the formula



in which R<sup>b</sup> denotes a hydrogen atom, is reacted with piperazine or a piperazine derivative of the formula



in which

R<sup>a</sup> has the meaning given above, or

(b') a compound of the formula (II'), as given in reaction variant (a) in which R<sup>b</sup> denotes an alkyl group, is reacted with a compound of formula (III') as defined in reaction variant (a), if appropriate, in the presence of an

acid-binding agent (such as triethylamine, 1,4-diazabicyclo[2,2,2]octane or 1,8-diazabicyclo[5,4,0]undec-7-ene) and the 7-piperazino-quinolone-3-carboxylic acid ester obtained is hydrolysed under alkaline conditions to give a compound of formula (I'),

and the compound of formula (I') obtained by reaction variant (a) or (b) is converted, if desired, into a salt and/or a hydrate thereof.

The reaction variant (a) is preferably carried out in a diluent (such as dimethylsulphoxide, N,N-dimethylformamide, hexamethyl-phosphoric acid triamide, sulfolane, water, an alcohol or pyridine) and at a temperature between 20° and 200° C., preferably between 80° and 180° C.

The reaction variants can be carried out under normal pressure, but also under elevated pressure, in particular in the case of a low-boiling solvent. In general, the reaction is carried out under pressures between about 1 and about 100 bar, preferably between 1 and 10 bar.

In carrying out reaction variants 1 to 5 mol of alkylpiperazine (in the case of piperazine 1 to 15 mol), preferably 2 to 3 mol of alkylpiperazine (in the case of piperazine 5 to 10 mol), are employed per mol of carboxylic acid, or carboxylic acid ester of formula (II').

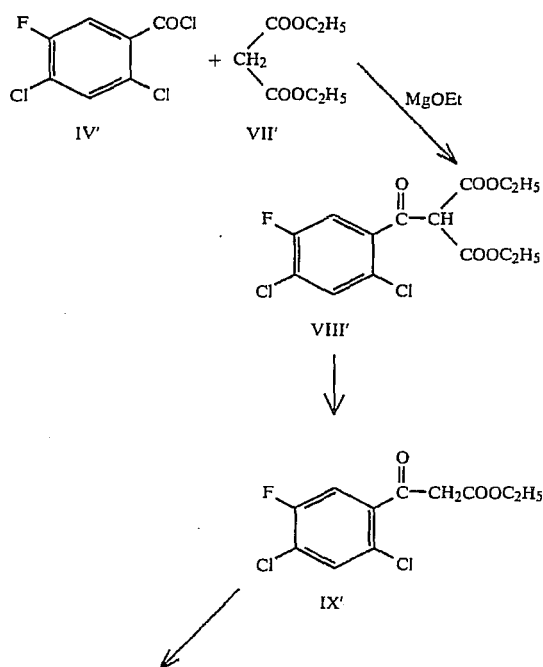
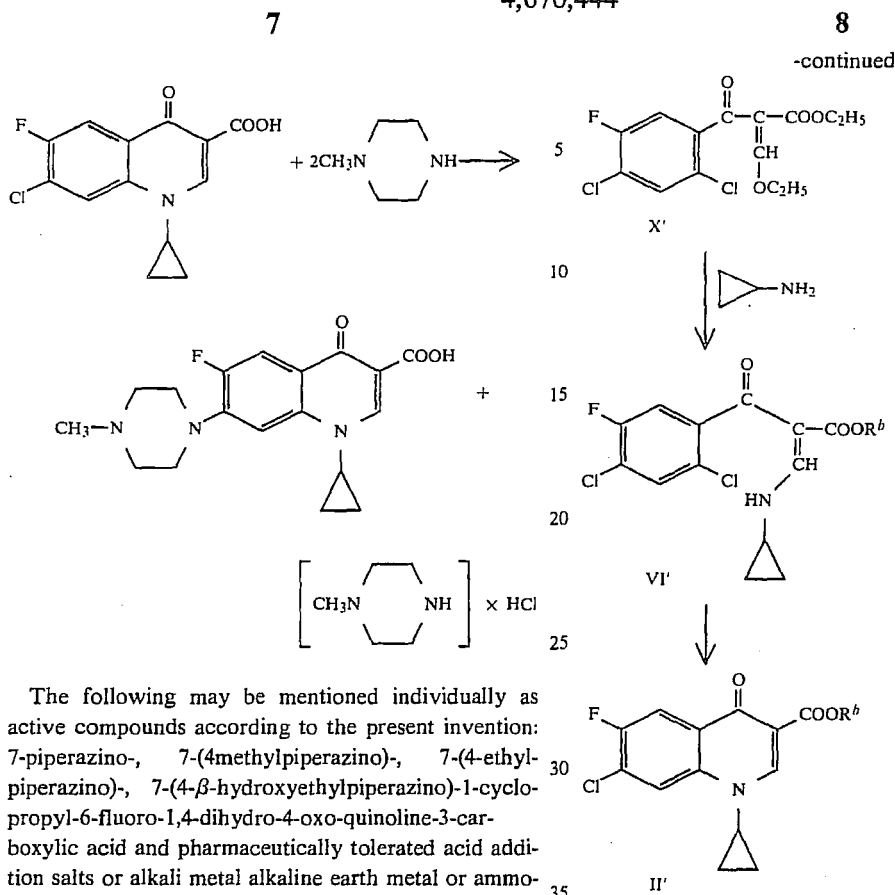
Among the new 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acid salts and hydrates of the invention those salts or hydrates that are pharmaceutically acceptable are particularly important and are preferred.

The new free 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acids of the general formula (I) and their salts and hydrates can be interconverted in any suitable manner; methods for such interconversion are known in the art.

Thus the 7-piperazino-quinolone-3-carboxylic acids of formula (I) obtained can, if required, be converted into a salt using an organic or inorganic acid. Examples of acids which are suitable for salt formation are hydrohalic acids, such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulphuric acid, acetic acid, citric acid and benzenesulphonic acid.

If 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid and methylpiperazine are used as starting materials in reaction variant (a), the course of the reaction is illustrated by the following equation:

4,670,444



According to this equation, diethyl malonate of formula (VII) is acylated with a compound of formula (IV') in the presence of magnesium alcoholate to give the acylmalonate of formula (VIII) (Organicum, 3rd edition 1964, page 438).

The ethyl aroylacetate of formula (IX') is obtained in good yield by partial hydrolysis and decarboxylation of the compound of formula (VIII') in an aqueous medium containing a catalytic amount of p-toluenesulphonic acid, and is converted with triethyl o-formate/acetic anhydride into the ethyl 2-(2,4-dichloro-5-fluorobenzoyl)-3-ethoxyacrylate of formula (X'). The reaction of the compound of formula (X') with cyclopropylamine in a solvent (such as methylene chloride, alcohol, chloroform, cyclohexane or toluene) leads to the desired intermediate product of formula (VI) in a slightly exothermic reaction.

The cyclisation reaction VI→II (R<sup>1</sup>=alkyl) is carried out in a temperature range of 60° to 280° C., preferably 80° to 180° C.

Dioxane, dimethylsulphoxide, N-methyl-pyrrolidone, sulfolane, hexamethylphosphoric acid triamide and preferably N,N-dimethylformamide can be used as diluents.

Potassium t-butanolate, butyl-lithium, lithium-phenyl, phenyl magnesium bromide, sodium ethylate and particularly preferably sodium hydride or potassium carbonate are suitable acid-binding agents for this reaction stage. It can be advantageous to employ an excess of 10 mol% of base.

The 2,4-dichloro-5-fluorobenzoyl chloride of formula (IV) used as a starting material for this

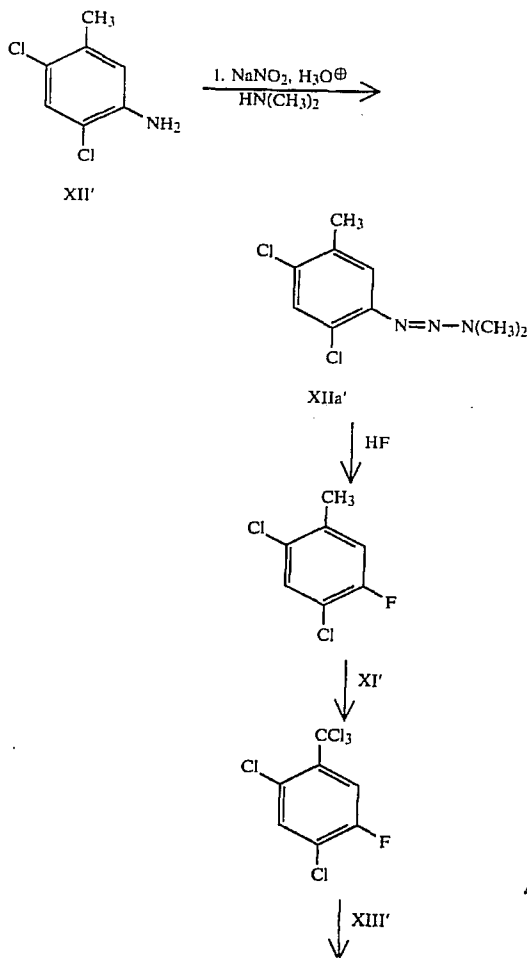


9

4,670,444

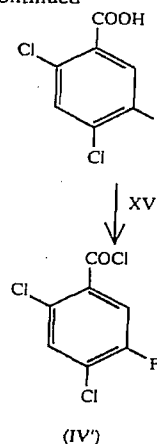
synthesis route, the corresponding carboxylic acid, and the 3-fluoro-4,6-dichlorotoluene of formula (XI) required for the preparation of formula (IV) were not yet known in the literature and form a further subject of the present invention.

The equation below shows the preparation of these precursors or intermediate products, starting from 2,4-dichloro-5-methyl-aniline of formula (XII').



10

-continued



According to this equation, 2,4-dichloro-5-methyl-aniline of formula (XII') is diazotised by means of  $\text{NaNO}_2$ , and the resulting diazonium salt is converted into the triazene of formula (XIIa'), using dimethylamine.

Triazene of formula (XIIa') is dissolved in excess anhydrous  $\text{HF}$ . In this step, the triazene is cleaved to give 2,4-dichloro-5-methyl-diazonium fluoride and dimethylamine. Without intermediate isolation, this solution is cleaved thermally at  $130^\circ$  to  $140^\circ$  to give 3-fluoro-4,6-dichlorotoluene XI',  $\text{N}_2$  being split off (Yield: 77.7% of theory).

The 3-fluoro-4,6-dichlorotoluene of formula (XI') is chlorinated in a temperature range from  $110^\circ$  to  $160^\circ \text{C.}$ , under UV irradiation, to give 2,4-dichloro-5-fluoro-1-trichloro-methylbenzene of formula (XIII').

The hydrolysis of the compound of formula (XIII') with 95 percent sulphuric acid leads to 2,4-dichloro-5-fluoro-benzoic acid of formula (XV'), which is converted with thionyl chloride into the carboxylic acid-chloride of formula (IV').

The compounds according to the invention are distinguished by a particularly good antibacterial action against gram positive and gram negative bacteria, in particular in comparison with the compounds of German Patent Application No. P 30 33 157.8 of 3.9.1980 and DE-OS (German Published Specification) No. 2,804,097, as can be seen from the table below.

	Example 2 of German Patent Application P 30 33 157.8 of 3.9.80	(disclosed in DE-OS (German Published Specification) 2,804,097)	(compound according to the invention, of the formula I (R = H))
<i>Staphylococcus aureus</i> 133	8	1	0.25-0.5
<i>E. coli</i> A 261	1	0.125	0.06
<i>E. coli</i> Neum.	1	0.25	0.06
<i>Klebsiella</i>	1	0.25	0.06



-continued

8085			
Proteus	0.5	0.06	0.03
1017			
<i>Pseudo-</i>	4	1	0.5
<i>monas</i>			
<i>aeruginosa</i>			
W			

Agar dilution test

DST (dextrose sensitivity test) medium:  $1-2 \times 10^3$  germs/plate

New antibacterial active compounds which may be mentioned specifically are: 7-methylamino-, 7-benzylamino-, 7-pyrrolidino-, 7-morpholino-, 7-piperidino-, 7-piperazino-, 7-(4-methylpiperazino)-, 7-(4-benzylpiperazino)-, 7-(4- $\beta$ -hydroxyethylpiperazino)-, 7-(4- $\gamma$ -hydroxypropylpiperazino)-, 7-(4-formylpiperazino)- or 7-(4-hydroxypiperidino)-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid and pharmaceutically acceptable acid addition salts or alkali or alkaline earth metal salts of these compounds.

It has furthermore been found that the compounds according to the invention have outstanding antimicrobial properties.

In particular, they have a broad bacteriostatic and bactericidal action against Gram-positive bacteria, such as Staphylococci and Streptococci, and Gram-negative bacteria, such as Escherichia, Proteus, Providencia, Enterobacter, Klebsiella, Salmonella and Pseudomonas. The list of sensitive bacteria is to be regarded as a list of examples and in no way restrictive.

The improved broad antibacterial activity of the compounds according to the invention enable them to be used as active compounds both in medicine, in which they can be used both for preventing and for the treatment of systemic or local bacterial infections, in particular of the urinary tract. The compounds according to the invention can furthermore also be used as feed additives for promoting growth and for improving feed utilisation in livestock husbandry, in particular in the rearing of animals for fattening. The active compounds are then preferably administered via the feed and/or the drinking water.

The present invention furthermore relates to agents which contain the new compounds according to the invention. These agents include, for example, feed concentrates, for livestock husbandry, which can also contain, as is customary, vitamins and/or mineral salts, in addition to the active compounds, and pharmaceutical formulations.

Among the new 7-amino-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acid salts of the invention, those salts that are pharmaceutically acceptable are particularly important and are preferred, alkali metal salts and alkaline earth metal salts being particularly preferred.

The new free 7-amino-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-carboxylic acids of the general formula (I) and (I') and their salts can be interconverted in any suitable manner; methods for such interconversion are known in the art.

As stated above, the invention also relates to the use in medicine of the compounds of the invention.

The present invention provides a pharmaceutical composition containing as active ingredient a compound of the invention in admixture with an inert pharmaceutical carrier, e.g. a solid or liquefied gaseous diluent, or in admixture with a liquid diluent other than a solvent of a molecular weight less than 200 (preferably

less than 350) except in the presence of a surface active agent.

The invention further provides a pharmaceutical composition containing as active ingredient a compound of the invention in the form of a sterile and/or physiologically isotonic aqueous solution.

The invention also provides a medicament in dosage unit form comprising a compound of the invention.

The invention also provides a medicament in the form of tablets (including lozenges and granules), dragees, capsules, pills, ampoules or suppositories comprising a compound of the invention.

"Medicament" as used in this Specification means physically discrete coherent portions suitable for medical administration. "Medicament in dosage unit form" as used in this Specification means physically discrete coherent units suitable for medical administration each containing a daily dose or a multiple (up to four times) or submultiple (down to a fortieth) of a daily dose of the compound of the invention in association with a carrier and/or enclosed within an envelope. Whether the medicament contains a daily dose or, for example, a half, a third or a quarter of a daily dose will depend on whether the medicament is to be administered once or, for example, twice, three times or four times a day respectively.

The pharmaceutical composition according to the invention may, for example, take the form of ointments, gels, pastes, creams, sprays (including aerosols), lotions, suspensions, solutions and emulsions of the active ingredient in aqueous or non-aqueous diluents, syrups, granulates or powders.

The diluents to be used in pharmaceutical compositions (e.g. granulates) adapted to be formed into tablets, dragees, capsules and pills include the following: (a) fillers and extenders, e.g. starch, sugars, mannitol, and silicic acid; (b) binding agents, e.g. carboxymethyl cellulose and other cellulose derivatives, alginates, gelatine and polyvinyl pyrrolidone; (c) moisturizing agents, e.g. glycerol; (d) disintegrating agents, e.g. agar-agar, calcium carbonate and sodium bicarbonate; (e) agents for retarding dissolution e.g. paraffin; (f) resorption accelerators, e.g. quaternary ammonium compounds; (g) surface active agents, e.g. cetyl alcohol, glycerol monostearate; (h) adsorptive carriers, e.g. kaolin and bentonite; (i) lubricants, e.g. talc, calcium and magnesium stearate and solid polyethyl glycols.

The tablets, dragees, capsules and pills formed from the pharmaceutical compositions of the invention can have the customary coatings, envelopes and protective matrices, which may contain opacifiers. They can be so constituted that they release the active ingredient only or preferably in a particular part of the intestinal tract, possibly over a period of time. The coatings, envelopes and protective matrices may be made, for example, of polymeric substances or waxes.

4,670,444

13

The ingredient can also be made up in microencapsulated form together with one or several of the above-mentioned diluents.

The diluents to be used in pharmaceutical compositions adapted to be formed into suppositories can, for example, be the usual water-soluble diluents, such as polyethylene glycols and fats (e.g. cocoa oil and high esters (e.g. C<sub>14</sub>-alcohol with C<sub>16</sub>-fatty acid)) or mixtures of these diluents.

The pharmaceutical compositions which are ointments, pastes, creams and gels can, for example, contain the usual diluents, e.g. animal and vegetable fats, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide or mixtures of these substances.

The pharmaceutical compositions which are powders and sprays can, for example, contain the usual diluents, e.g. lactose, talc, silicic acid, aluminium hydroxide, calcium silicate, and polyamide powder or mixtures of these substances. Aerosol sprays can, for example, contain the usual propellants, e.g. chlorofluorohydrocarbons.

The pharmaceutical compositions which are solutions and emulsions can, for example, contain the customary diluents (with, of course, the above-mentioned exclusion of solvents having a molecular weight below 200 except in the presence of a surface-active agent), such as solvents, dissolving agents and emulsifiers; specific examples of such diluents are water, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (for example ground nut oil), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitol or mixtures thereof.

For parenteral administration, solutions and emulsions should be sterile, and, if appropriate, blood-isotonic.

The pharmaceutical compositions which are suspensions can contain the usual diluents, such as liquid diluents, e.g. water, ethyl alcohol, propylene glycol, surface-active agents (e.g. ethoxylated isostearyl alcohols, polyoxyethylene sorbite and sorbitane esters), microcrystalline cellulose, aluminium metahydroxide, bentonite, agar-agar and tragacanth or mixtures thereof.

All the pharmaceutical compositions according to the invention can also contain colouring agents and preservatives as well as perfumes and flavouring additions (e.g. peppermint oil and eucalyptus oil) and sweetening agents (e.g. saccharin).

The pharmaceutical compositions according to the invention generally contain from 0.1 to 99.5% usually from 0.5 to 95% of the active ingredient by weight of the total composition.

In addition to a compound of the invention, the pharmaceutical compositions and medicaments according to the invention can also contain other pharmaceutically active compounds. They may also contain a plurality of compounds of the invention.

Any diluent in the medicaments of the present invention may be any of those mentioned above in relation to the pharmaceutical compositions of the present invention. Such medicaments may include solvents of molecular weight less than 200 as sole diluent.

The discrete coherent portions constituting the medicament according to the invention will generally be adapted by virtue of their shape or packaging for medical administration and may be, for example, any of the

14

following: tablets (including lozenges and granulates), pills, dragees, capsules, suppositories and ampoules. Some of these forms may be made up for delayed release of the active ingredient. Some, such as capsules, include a protective envelope which renders the portions of the medicament physically discrete and coherent.

The production of the above-mentioned pharmaceutical compositions and medicaments is carried out by any method known in the art, for example, by mixing the active ingredient(s) with the diluent(s) to form a pharmaceutical composition (e.g. a granulate) and then forming the composition into the medicament (e.g. tablets).

This invention further provides a method of combating the above-mentioned diseases in warm-blooded animals, which comprises administering to the animals a compound of the invention alone or in admixture with a diluent or in the form of a medicament according to the invention.

The provision of new bactericides for combating bacteria which are resistant to known bactericides as is the case with compounds of the present invention is an enrichment of the state of the art.

The following examples illustrated but do not limit the invention.

#### EXAMPLE 1

7-(4-Methylpiperazino)-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid (a compound of formula (I) in which R<sup>1</sup>R<sup>2</sup>N=4-methylpiperazino, A=N and B=CH).

A suspension of 2.64 g of 7-chloro-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid and 2.5 g of N-methylpiperazine in 30 ml of ethanol or DMSO (=Dimethylsulfoxide) was heated to the boiling point under reflux for 16 hours or to 135°-140° C. for two hours. The diluent was distilled off in vacuo, the residue was dissolved in 30 ml of 1N NaOH, the solution was filtered and the filtrate was acidified with 10 strength hydrochloric acid. The precipitate was filtered off and washed with water and ethanol. It could be recrystallised from N-dimethylformamide/ethanol. 3.1 g (94% of the theoretical yield) of 7-(4-methylpiperazino)-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid of melting point 326° C. (hydrochloride) (decomposition) were obtained.

#### EXAMPLES 2 TO 10

The carboxylic acids of Examples 2 to 19 were obtained by a procedure analogous to that in Example 1. They are summarised in Table 1. The labelling of the radicals R<sup>1</sup> and R<sup>2</sup> relates to the formula (I) of the description.

TABLE 1

Ex- ample No.	A	B	R <sup>1</sup>	R <sup>2</sup>	Decomposition Point (°C.)
2	N	CH	H	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —	322 (hydrochloride)
3	N	CH	—(CH <sub>2</sub> ) <sub>2</sub> O(CH <sub>2</sub> ) <sub>2</sub> —		286
4	N	CH	—(CH <sub>2</sub> ) <sub>2</sub> CH <sub>2</sub> (CH <sub>2</sub> ) <sub>2</sub> —		297
5	N	CH	—CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> —		330
6	N	CH	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   CH <sub>2</sub> CH <sub>2</sub> OH		305 (hydrochloride)

4,670,444

15

TABLE 1-continued

Ex- ample No.	A	B	R <sup>1</sup>	R <sup>2</sup>	Decomposition Point (°C.)
7	N	CH	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   (CH <sub>2</sub> ) <sub>3</sub> OH		(hydrochloride)
8	N	CH	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   CHO		300
9	N	CH	—CH <sub>2</sub> —CH—(CH <sub>2</sub> ) <sub>3</sub> —   OH		302
10	N	CH	—(CH <sub>2</sub> ) <sub>2</sub> CH(CH <sub>2</sub> ) <sub>2</sub> —   OH		279
1	CF	CH	H —(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   CH <sub>3</sub>		256 306 (hydrochloride)
2	CH	N	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   CH <sub>3</sub>		279
3	CH	N	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   H		277
4	CF	CH	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   CH <sub>3</sub>		249
5	CF	CH	—(CH <sub>2</sub> ) <sub>4</sub> —		323
6	C—CN	N	H —(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —		335 (hydrochloride)
7	C—CN	N	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   CH <sub>3</sub>		295 (hydrochloride)
8	C—CN	N	—(CH <sub>2</sub> ) <sub>4</sub> —		290
9	CF	CH	—(CH <sub>2</sub> ) <sub>2</sub> N(CH <sub>2</sub> ) <sub>2</sub> —   C <sub>2</sub> H <sub>5</sub>		306 (hydroiodide)

## EXAMPLE 20

## Preparation of precursors

(a) 6-Chloro-4-(N-2-methoxycarbonylethyl-N-cyclopropyl)-amino-pyridine-3-carboxylic acid methyl ester (a compound of formula (VI) in which R=methyl and X=chlorine).

A mixture of 28.6 g of  $\beta$ -cyclopropylamino-propionic acid methyl ester and 21 g of triethylamine was rapidly added dropwise to a solution of 41.2 g of 4,6-dichloropyridine-3-carboxylic acid methyl ester in 150 ml of toluene at 10° to 20° C., whilst cooling with ice and stirring. The ice-bath was removed and the mixture was stirred at room temperature for  $\frac{1}{2}$  hour and heated to the boiling point under reflux for 6 hours. The resulting suspension was washed with water and dried with Na<sub>2</sub>SO<sub>4</sub> and the solvent was distilled off in vacuo. 59 g of the title compound were obtained as a brown oil.

(b) The  $\beta$ -cyclopropylaminopropionic acid methyl ester

This compound, used as a reactant in Example 20(a), was prepared as follows:

86 g of freshly distilled methyl acrylate which had been cooled to -60° C. was added dropwise to a solution, which had been cooled to -60° C. to -70° C., of

16

57 g of cyclopropylamine in 150 ml of ethanol in the course of about 3 hours. The mixture was then allowed to rise slowly to room temperature overnight, the solvent was distilled off in vacuo and the residue was then fractionated. 95 g of  $\beta$ -cyclopropylamino-propionic acid methyl ester passed over at 84°-86° C./22 mm Hg.

(c) 7-Chloro-1-cyclopropyl-4-oxo-1,2,3,4-tetrahydro-1,6-naphthyridine-3-carboxylic acid methyl ester (a compound of formula (VII) in which R=methyl and X=chlorine).

59 g of crude 6-chloro-4-(N-2-methoxycarbonylethyl-N-cyclopropyl)-amino-pyridine-3-carboxylic acid methyl ester were dissolved in 240 ml of anhydrous toluene, and 23 g of potassium t-butyrate were rapidly added, whilst stirring. The mixture was left to stand overnight, 20 g of glacial acetic acid and 100 ml of water were added, the phases were separated, the toluene solution was washed again with water and dried with Na<sub>2</sub>SO<sub>4</sub> and the toluene was stripped off in vacuo. After recrystallisation from methanol, 18 g of the carboxylic acid ester of melting point 155° to 157° C. were obtained.

(d) 7-Chloro-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid methyl ester (a compound of formula (VIII) in which R=methyl and X=chlorine).

9.8 g of the tetrahydronaphthyridine-3-carboxylic acid methyl ester prepared according to Example 20(c) were dissolved in 200 ml of methylene chloride, and a solution of 5.9 g of bromine in 40 ml of CH<sub>2</sub>Cl<sub>2</sub> was rapidly added dropwise at 10° to 15° C., whilst cooling with ice. The mixture is then stirred at ~10° C. for a further 10 minutes, 8 g of triethylamine were added and the ice-bath was removed. The mixture was subsequently stirred for 3 hours, washed twice with water and dried with Na<sub>2</sub>SO<sub>4</sub>, the solvent was distilled off in vacuo and the residue was recrystallised from dimethyl-formamide/ethanol. 8.8 g of 7-chloro-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acid methyl ester of melting point 272° to 274° C. (decomposition) were obtained.

(e) 7-Chloro-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid (a compound of formula (II) in which R=H, A=N, Z=CH and X=chlorine).

A solution of 5.7 g of potassium hydroxide in 300 ml of water was added to 27.85 g of the ester prepared according to Example 11(d). The mixture was heated to 85° to 95° C. for 30 minutes, whilst stirring, and the resulting solution was filtered at room temperature and acidified with glacial acetic acid. The precipitate was filtered off, washed with water and dried over calcium chloride in a vacuum drying cabinet. 20 g of pure 7-chloro-1-cyclopropyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid of melting point 226° to 227° C. were obtained. (including prevention, relief and cure of the above-mentioned diseases in warm-blooded animals, which comprises administering to the animals a compound of the invention alone or in admixture with a diluent or in the form of a medicament according to the invention.

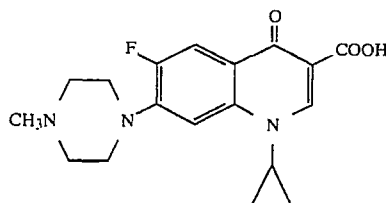
The present invention further provides a feed additive comprising an active compound of the present invention in admixture with a feed additive-carrier.

The Examples which follow illustrate the invention further.

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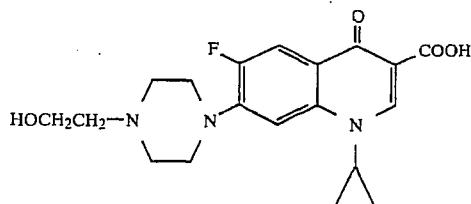
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## EXAMPLE 21



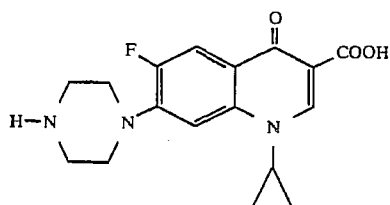
A mixture of 20 g of 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid, 28.5 g of N-methylpiperazine and 120 ml of anhydrous dimethylsulphoxide was heated at 135° to 140° C. for 1.5 hours. The solvent was distilled off under a fine vacuum, and the residue was suspended in approx. 50 ml of H<sub>2</sub>O. The suspension was filtered under suction, and the residue was rinsed with H<sub>2</sub>O, dried in a vacuum drying cabinet at 80° C. over CaCl<sub>2</sub>, and recrystallised from glycol monomethyl ether. 14.5 g of 1-cyclopropyl-6-fluoro-1,4-dihydro-7-(4-methylpiperazino)-4-oxo-quinoline-3-carboxylic acid which decomposes at 248° to 250° C. were obtained.

## EXAMPLE 22



A suspension of 2.81 of 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid and 5.2 g of N-β-hydroxyethylpiperazine in 25 ml of dimethylsulphoxide was heated at 135° to 140° C. for 2 hours. The solvent was distilled off under a fine vacuum, the residue was boiled for a short time with 20 ml of H<sub>2</sub>O and left to stand overnight at room temperature, and the precipitate was filtered off under suction, while cooling with ice, and was washed with water and dried in vacuo over CaCl<sub>2</sub> at 80° C. 2.1 g of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-β-hydroxyethylpiperazino)-quinoline-3-carboxylic acid which decomposed at 237° to 239° C. were obtained.

## EXAMPLE 23



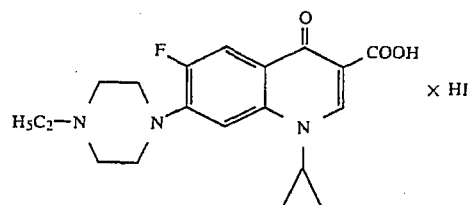
A mixture of 19.7 g of 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid, 30.1 g of anhydrous piperazine and 100 ml of dimethylsulphoxide was heated at 135° to 140° C. for 2 hours. The solvent was distilled off under a fine vacuum, and the residue was suspended in H<sub>2</sub>O, filtered off under

18

suction and washed with water. For further purification, the moist crude product was boiled with 100 ml of water, filtered off under suction at room temperature, washed with H<sub>2</sub>O and dried over CaCl<sub>2</sub> in a vacuum drying cabinet at 100° C. until its weight remained constant. 19.6 g of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acid which decomposed at 255° to 257° C. were obtained.

The compound prepared according to Example 3 was dissolved in 50 of hot 10 percent hydrochloric acid. 150 ml of ethanol were added to the filtered solution, the mixture was cooled with ice, and the product was filtered off under suction, washed with alcohol, and dried in vacuo at 100° C. 18.5 g of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acid hydrochloric were obtained as colourless crystals which decomposed at 326°-328° C. The monohydrate of this hydrochloride has a m.p. 318°-320° C.

## EXAMPLE 24



(a) A mixture of 1.2 g of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acid, 1.13 g of ethyl iodide, 0.73 g of triethylamine and 20 ml of N,N-dimethylformamide was heated at 70° to 80° C. for 2.5 hours. The solvent was distilled off in vacuo, and the residue was suspended in water. The product was filtered off under suction, rinsed with H<sub>2</sub>O and pressed on clay. 1.15 g of 1-cyclopropyl-6-fluoro-7-(ethylpiperazino)-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid hydroiodide which decomposes at 306° C. were obtained.

(b) The 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid used as the starting material was prepared as follows:

24.3 g of magnesium turnings were suspended in 50 ml of anhydrous ethanol. 5 ml of carbon tetrachloride were added and, when the reaction had started, a mixture of 160 g of diethyl malonate, 100 ml of absolute ethanol and 400 ml of anhydrous ether was added dropwise, a vigorous reflux being observed. After the reaction had ceased, the mixture was heated at the boil for a further 2 hours and was cooled with dry ice/acetone at -5° C. to -10° C. and a solution of 227.5 g of 2,4-dichloro-5-fluoro-benzoyl chloride in 100 ml of absolute ether was slowly added dropwise at this temperature. The mixture was stirred for 1 hour at 0° C. to -5° C. and was allowed to reach room temperature overnight, and a mixture of 400 ml of ice-water and 25 ml of concentrated sulphuric acid was allowed to run in while cooling with ice. The phases were separated and were extracted twice with ether. The combined ether solutions were washed with saturated NaCl solution and dried with Na<sub>2</sub>SO<sub>4</sub>, and the solvent was stripped off in vacuo. 349.5 g of diethyl 2,4-dichloro-5-fluoro-benzoyl-malonate were obtained as the crude product.

0.15 g of p-toluenesulphonic acid was added to an emulsion of 34.9 g of crude diethyl 2,4-dichloro-5-



4,670,444

19

fluoro-benzoyl-malonate in 50 ml of water. The emulsion was heated at the boil for 3 hours while stirring thoroughly, and, when cold, was extracted several times with methylene chloride, the combined  $\text{CH}_2\text{Cl}_2$  solutions were washed once with saturated NaCl solution and dried with  $\text{Na}_2\text{SO}_4$ , and the solvent was distilled off in vacuo. Fractionation of the residue under a fine vacuum gave 21.8 g of ethyl 2,4-dichloro-5-fluorobenzoyl acetate IX of boiling point  $127^\circ$  to  $142^\circ$  C./0.09 mbar.

A mixture of 21.1 g of ethyl 2,4-dichloro-5-fluorobenzoyl-acetate, 16.65 g of ethyl o-formate and 18.55 g of acetic anhydride was heated at  $150^\circ$  C. for 2 hours. The volatile constituents were then distilled off under a waterjet vacuum and finally under a fine vacuum, at a bath temperature of  $120^\circ$  C. 25.2 g of crude ethyl 2-(2,4-dichloro-5-fluoro-benzoyl)-3-ethoxy-acrylate remained. It was sufficiently pure for the further reactions.

4.3 g of cyclopropylamine were added dropwise to a solution of 24.9 g of ethyl 2-(2,4-dichloro-5-fluorobenzoyl)-3-ethoxy-acrylate in 80 ml of ethanol while cooling with ice and stirring. When the exothermic reaction had ceased, the mixture was stirred for another hour at room temperature, the solvent was stripped off in vacuo, and the residue was recrystallised from cyclohexane/petroleum ether. 22.9 g of ethyl 2-(2,4-dichloro-5-fluoro-benzoyl)-3-cyclopropylamino-acrylate ( $\text{R}^1 = \text{C}_2\text{H}_5$ ) of melting point  $89^\circ$  to  $90^\circ$  C. were obtained.

3.44 g of 80 percent sodium hydride were added in portions to a solution of 31.9 g of ethyl 2-(2,4-dichloro-5-fluoro-benzoyl)-3-cyclopropylamino-acrylate ( $\text{R}^1 = \text{C}_2\text{H}_5$ ) in 100 ml of anhydrous dioxane while cooling with ice and stirring. The mixture was then stirred at room temperature for 30 minutes and under reflux for 2 hours, and the dioxane was stripped off in vacuo. The residue (40.3 g) was suspended in 150 ml of water, 6.65 g of caustic potash were added, and the mixture was refluxed for 1.5 hours. The warm solution was filtered and the residue was rinsed with  $\text{H}_2\text{O}$ . The filtrate was then acidified to pH=1 to 2 with semiconcentrated hydrochloric acid, while cooling with ice, and the precipitate was filtered off under suction, washed with water and dried in vacuo at  $100^\circ$  C. 27.7 g of 7-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid ( $\text{R}^1 = \text{H}$ ) of melting point  $234^\circ$  to  $237^\circ$  C. were obtained in this manner.

The present invention also comprises pharmaceutically acceptable bioprecursors of the active compounds of the present invention.

The following example shows the recipe of a tablet according to the invention:

1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-3-carboxylic acid HCl	277.5 mg	(corresponding to 250.0 mg Butain)
Avicel	49.0 mg	
Moist corn starch	14.0 mg	
Pregelatinized starch	6.0 mg	
Magnesium stearate	3.5 mg	
tablet without film coating	350.0 mg	
Film coating		
HPM cellulose 15 cp	3.0 mg	
Polyethylene glycol 4000	1.0 mg	
Titanium dioxide	1.0 mg	
film coated tablet	355.0 mg	

20

For the purpose of this specification the term "pharmaceutically acceptable bioprecursor" of an active compound of the invention means a compound having a structural formula different from the active compound but which nonetheless, upon administration to a warm-blooded animal is converted in the patient's body to the active compound.

The improved bacterial action of the compounds of Example 1 according to the present invention is particularly clear in the following biotest Example, in which it was compared with 2-piperazino-8-ethyl-5-oxo-5,8-dihydropyrido 2,3-d pyrimidine-6-carboxylic acid ("pipemidic acid") or the known compound 1-ethyl-7-methyl-1,8-naphthyrid-4-one-3-carboxylic acid ["nalidixic acid"; Ehrhart/Ruschig, Arzneimittel (Medicaments), Volume 2: Chemotherapeutika (Chemotherapeutics), Verlag Chemie 1968, page 1,568]. The compounds of the invention have proved to be far superior in vitro and in vivo on bacteria such as Staphylococci, *Escherichia coli*, Proteus, Klebsiella and Pseudomonas than such known compounds.

#### EXAMPLE

The agar dilution test was carried out by the Denley multipoint inoculation method and the results were as shown in the following Table.

	Minimum inhibitory concentrations mog/ml in an agar dilution test <sup>x</sup>		
	Compounds from Example 1	Pipemidic acid	Nalidixic acid
<i>Escherichia coli</i>			
T 7	0.25	2	1
455/7	128	128	256
103400	0.25	1	2
Salmonella 683	0.5	2	4
Klebsiella 63	1	2	4
Pseudomonas 7167	8	16	64
Proteus 8228	2	4	8

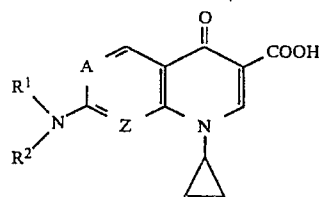
<sup>x</sup>Denley multipoint inoculation method

The present invention also comprises pharmaceutically acceptable bioprecursors of the active compounds of the present invention.

For the purposes of this specification the term 'pharmaceutically acceptable bioprecursor' of an active compound of the invention means a compound having a structural formula different from the active compound but which nonetheless, upon administration to a warm-blooded animal is converted in the patient's body to the active compound.

What is claimed is:

1. A compound which is a 7-amino-1-cyclopropyl-4-oxo-1,4-dihydro-quinoline- and -naphthyridine-3-carboxylic acid of the formula



(I)

or a pharmaceutically acceptable acid addition salt or an alkali or alkaline earth metal salt thereof, in which A represents a nitrogen atom or  $\text{CR}^3$ , wherein  $\text{R}^3$  denotes a hydrogen, a nitro group or a halogen atom, or a carboxamide or carboxyl group, and

Z represents a nitrogen atom or  $\text{C}-\text{H}$ , and A and Z cannot simultaneously be nitrogen atoms, and  $\text{R}^1$

21

4,670,444

and R<sup>2</sup> are identical or different and represent a hydrogen atom or a straight-chain or branched alkyl, alkenyl or alkynyl radical which has up to 12 carbon atoms and is optionally substituted by radical(s) selected from hydroxyl, alkoxy, alkylmercapto or dialkylamino with 1 to 3 carbon atoms in each alkyl radical, alkoxycarbonyl with 1 to 4 carbon atoms in the alcohol part, and mono- or bi-cyclic carbocyclic aryl, or furthermore represents a cycloalkyl radical with 3 to 6 carbon atoms, or, together with the nitrogen atom which they substituted or together with a further hetero-atom selected from the group consisting of N, O and S form a 3-membered to 7-membered ring which can be substituted by radical(s) selected from alkyl or alkenyl with 1 to 6 carbon atoms, hydroxyl, alkoxy or alkylmercapto with 1 to 3 carbon atoms, alkoxycarbonyl with 1 to 4 carbon atoms in the alcohol part, and mono- or bi-cyclic carbocyclic aryl.

2. A compound according to claim 1, in which A represent CR<sup>3</sup> and R<sup>3</sup> represents a fluorine or chlorine atom.

3. A compound according to claim 1 or 2, in which R<sup>1</sup> and R<sup>2</sup> together with the nitrogen atom which they substituted and oxygen, sulphur or R<sup>4</sup>-substituted nitrogen as a further hetero-atom form a 3-membered or 7-membered ring which may be substituted by radical(s) selected from alkyl or alkenyl with up to 6 carbon atoms, hydroxyl, alkoxy or alkylmercapto with 1 to 3 carbon atoms, alkoxycarbonyl with 1 to 4 carbon atoms in the alcohol part, and mono- or bi-cyclic carboxylic aryl,

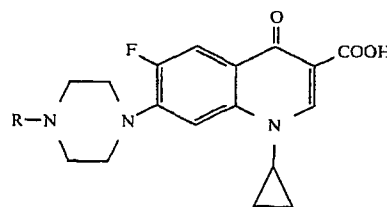
and in which R<sup>4</sup> represents a hydrogen, or an unsubstituted branched or straight-chain alkyl group which has up to 6 carbon atoms or a branched or straight-chain alkyl which has up to 6 carbon atoms which is substituted by radicals(s) selected from hydroxyl, alkoxy, alkylmercapto or dialkylamino with 1 to 3 carbon atoms per alkyl radical, and alkoxycarbonyl with 1 to 4 carbon atoms in the alcohol part, or represents an phenylalkyl group which has up to 4 carbon atoms in the aliphatic part, or an optionally substituted phenyl or naphthyl group or pyridine, pyrimidine, thiazole or benzothiazole, or

R<sup>4</sup> denotes an alkoxycarbonyl group which is optionally substituted by a mono- or bi-cyclic carbocyclic aryl radical and has 1 to 4 carbon atoms in an alcohol part an alkanoyl radical with 1 to 6 carbon atoms, a benzoyl or naphthoyl radical, an alkyl-, phenyl- or naphthyl-(thio) carbamoyl radical, an alkyl-, phenyl- or naphthyl-sulphonyl radical or an amino-sulphonyl radical.

4. A compound according to claim 3, in which R<sup>4</sup> represents a radical of pyridine, pyrimidine, thiazole or benzothiazole.

5. A 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acid of the formula

22



or salts and/or hydrates thereof,

in which R denotes hydrogen, methyl, ethyl or  $\beta$ -hydroxyethyl.

6. A compound according to claim 1 which is 7-(4-methylpiperazino)-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acid.

7. A compound according to claim 1 which is 7-piperazino-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acid.

8. A compound according to claim 1 which is 7-pyrrolidino-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acid.

9. A compound according to claim 1 which is 7-(4-formylpiperazino)-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acid.

10. A compound according to claim 1 which is 7-(4-hydroxyethylpiperazino)-1-cyclopropyl-4-oxo-1,4-dihydro-naphthyridine-3-carboxylic acid.

11. A compound according to claim 1 which is 7-piperazino-1-cyclopropyl-4-oxo-1,4-dihydro-6-fluoro-quinoline-3-carboxylic acid.

12. A compound of claim 5 which is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazino-quinoline-3-carboxylic acid.

13. A compound of claim 5 which is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-methylpiperazino)-quinoline-3-carboxylic acid.

14. A compound of claim 5 which is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-ethylpiperazino)-quinoline-3-carboxylic acid.

15. A compound of claim 5 which is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4- $\beta$ -hydroxyethylpiperazino)-quinoline-3-carboxylic acid.

16. A pharmaceutical composition containing as an active ingredient an antibacterially effective amount of a compound according to claim 1 in admixture with an inert pharmaceutical carrier.

17. A pharmaceutical composition according to claim 16 in the form of a sterile or physiologically isotonic aqueous solution.

18. A composition according to claim 16 or 17 containing from 0.5 to 95% by weight of the said active ingredient.

19. A medicament in dosage unit form comprising an antibacterially effective amount of a compound according to claim 1 together with an inert pharmaceutical carrier.

20. A medicament of claim 18 in the form of tablets, pills, dragees, capsules, ampoules, or suppositories.

21. A method of combating bacterial illnesses in warm-blood animals which comprises administering to the animals an antibacterially effective amount of an active compound according to claim 1 either alone or in admixture with a diluent or in the form of a medicament.

22. An animal feed, food concentrate or drinking water comprising an active compound according to claim 1.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 4,670,444

Page 1 of 2

DATED : June 2, 1987

INVENTOR(S) : Klaus Grohe, et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 2, line 7	After "preferably 1" insert --or--
Col. 2, line 15	Delete "aryl" and substitute --aroyl--
Col. 2, lines 34-35	Correct spelling of --monosubsti- tuted--
Col. 4, line 34	Delete "(IV)" and substitute --(VI)--
Col. 8, line 67	Delete "5-dichloro--"
Col. 9, line 8	Delete "(XIII)" and substitute --(XII)--
Col. 10, line 28	After "methyl" delete ":" and substitute -- - --
Col. 13, line 34	Correct spelling of --sorbitol--
Col. 15, Table 1 con't, last column heading	Delete "(°C)" and substitute --(°C)--
Col. 15, Table 1 con't Ex. No. 7, last column	Insert --306-- above "(hydro- chloride)"
Col. 15, Table 1 con't	Delete Example Nos. "1 to 9" and substitute --11 to 19--
Col. 18, line 10	After "50" insert --ml--
Col. 18, line 17	Delete "hydrochloric" and sub- stitute --hydrochloride--
Col. 20, line 1	Delete "purpose" and substitute --purposes--

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 4,670,444

Page 2 of 2

DATED : June 2, 1987

INVENTOR(S) : Klaus Grohe, et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 21, line 36

Delete "carboxylic" and substitute  
--carbocyclic--

Col. 21, line 56

Before "alcohol" delete "a" and  
substitute --the--

Signed and Sealed this  
Fifteenth Day of March, 1988

*Attest:*

DONALD J. QUIGG

*Attesting Officer*

*Commissioner of Patents and Trademarks*



**EXHIBIT B**

**CERTIFICATE EXTENDING**

**PATENT TERM**

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE EXTENDING PATENT TERM  
UNDER 35 U.S.C. § 156

PATENT NO. : 4,670,444  
ISSUED : June 2, 1987  
INVENTOR(S) : Klaus Grohe et al.  
PATENT OWNER : Bayer Aktiengesellschaft

This is to certify that there has been presented to the

COMMISSIONER OF PATENTS AND TRADEMARKS

an application under 35 U.S.C. § 156 for an extension of the patent term. Since it appears that the requirements of the law have been met, this certificate extends the term of the patent for the period of

Three years

from December 9, 2003, the original expiration date of the patent, subject to the provisions of 35 U.S.C. § 41(b), with all rights pertaining thereto as provided by 35 U.S.C. § 156(b).



I have caused the seal of the Patent and Trademark Office to be affixed this 15th day of July 1998.

*Bruce A. Lehman*

Bruce A. Lehman  
Assistant Secretary of Commerce and  
Commissioner of Patents and Trademarks

# EXHIBIT C

## CIPRO<sup>®</sup> LABELS

*(Cipro<sup>®</sup> HC p. 4 of 5; Cipro<sup>®</sup> HC OTIC p. 4 of 5)*

Your Search Terms: PATENT

Version 1 - Published Mar 13, 2008

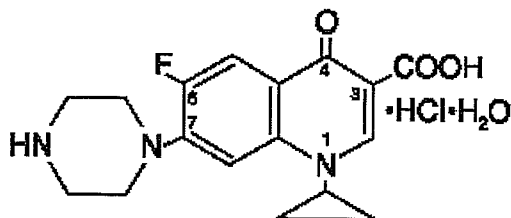
**CIPRO - ciprofloxacin hydrochloride, hydrocortisone and benzyl alcohol suspension**  
Alcon

**CIPRO® HC OTIC**  
(ciprofloxacin hydrochloride and  
hydrocortisone otic suspension)

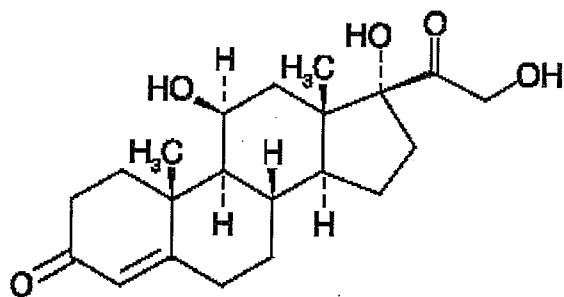
**DESCRIPTION**

CIPRO® HC OTIC (ciprofloxacin hydrochloride and hydrocortisone otic suspension) contains the synthetic broad spectrum antibacterial agent, ciprofloxacin hydrochloride, combined with the anti-inflammatory corticosteroid, hydrocortisone, in a preserved, nonsterile suspension for otic use. Each mL of CIPRO® HC OTIC contains ciprofloxacin hydrochloride (equivalent to 2 mg ciprofloxacin), 10 mg hydrocortisone, and 9 mg benzyl alcohol as a preservative. The inactive ingredients are polyvinyl alcohol, sodium chloride, sodium acetate, glacial acetic acid, phospholipon 90H (modified lecithin), polysorbate, and purified water. Sodium hydroxide or hydrochloric acid may be added for adjustment of pH.

Ciprofloxacin, a fluoroquinolone, is available as the monohydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. Its empirical formula is  $C_{17}H_{18}FN_3O_3 \cdot HCl \cdot H_2O$  and its chemical structure is as follows:



Hydrocortisone, pregn-4-ene-3, 20-dione, 11, 17, 21-trihydroxy-(11 $\beta$ )-, is an anti-inflammatory corticosteroid. Its empirical formula is  $C_{21}H_{30}O_5$  and its chemical structure is:



**CLINICAL PHARMACOLOGY**

The plasma concentrations of ciprofloxacin were not measured following three drops of otic suspension administration because the systemic exposure to ciprofloxacin is expected to be below the limit of quantitation of the assay (0.05  $\mu\text{g/mL}$ ).

Similarly, the predicted C<sub>max</sub> of hydrocortisone is within the range of endogenous hydrocortisone concentration (0-150 ng/mL), and therefore can not be differentiated from the endogenous cortisol.

Preclinical studies have shown that CIPRO<sup>®</sup> HC OTIC was not toxic to the guinea pig cochlea when administered intratympanically twice daily for 30 days and was only weakly irritating to rabbit skin upon repeated exposure.

Hydrocortisone has been added to aid in the resolution of the inflammatory response accompanying bacterial infection.

#### Microbiology

Ciprofloxacin has in vitro activity against a wide range of gram-positive and gram-negative microorganisms. The bactericidal action of ciprofloxacin results from interference with the enzyme, DNA gyrase, which is needed for the synthesis of bacterial DNA. Cross-resistance has been observed between ciprofloxacin and other fluoroquinolones. There is generally no cross-resistance between ciprofloxacin and other classes of antibacterial agents such as beta-lactams or aminoglycosides.

Ciprofloxacin has been shown to be active against most strains of the following microorganisms, both in vitro and in clinical infections of acute otitis externa as described in the **INDICATIONS AND USAGE** section:

#### **Aerobic gram-positive microorganism**

*Staphylococcus aureus*

#### **Aerobic gram-negative microorganisms**

*Proteus mirabilis*

*Pseudomonas aeruginosa*

#### **INDICATIONS AND USAGE**

CIPRO<sup>®</sup> HC OTIC is indicated for the treatment of acute otitis externa in adult and pediatric patients, one year and older, due to susceptible strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Proteus mirabilis*.

#### **CONTRAINDICATIONS**

CIPRO<sup>®</sup> HC OTIC is contraindicated in persons with a history of hypersensitivity to hydrocortisone, ciprofloxacin or any member of the quinolone class of antimicrobial agents. This nonsterile product should not be used if the tympanic membrane is perforated. Use of this product is contraindicated in viral infections of the external canal including varicella and herpes simplex infections.

#### **WARNINGS**

#### **NOT FOR OPHTHALMIC USE. NOT FOR INJECTION.**

CIPRO<sup>®</sup> HC OTIC should be discontinued at the first appearance of a skin rash or any other sign of hypersensitivity. Serious and occasionally fatal hypersensitivity (anaphylactic) reactions, some following the first dose, have been reported in patients receiving systemic quinolones. Serious acute hypersensitivity reactions may require immediate emergency treatment.

#### **PRECAUTIONS**

##### **GENERAL**

As with other antibiotic preparations, use of this product may result in overgrowth of nonsusceptible organisms, including fungi. If the infection is not improved after one week of therapy, cultures should be obtained to guide further treatment.

##### **Information for Patients**

If rash or allergic reaction occurs, discontinue use immediately and contact your physician.

Do not use in the eyes.

Avoid contaminating the dropper with material from the ear, fingers, or other sources.

Protect from light.

Shake well immediately before using.

Discard unused portion after therapy is completed.

#### Carcinogenesis, Mutagenesis, Impairment of Fertility

Eight *in vitro* mutagenicity tests have been conducted with ciprofloxacin, and the test results are listed below:

Salmonella/Microsome Test (Negative)

*E. coli* DNA Repair Assay (Negative)

Mouse Lymphoma Cell Forward Mutation Assay (Positive)

Chinese Hamster V79 Cell HGPRT Test (Negative)

Syrian Hamster Embryo Cell Transformation Assay (Negative)

*Saccharomyces cerevisiae* Point Mutation Assay (Negative)

*Saccharomyces cerevisiae* Mitotic Crossover and Gene Conversion Assay (Negative)

Rat Hepatocyte DNA Repair Assay (Positive)

Thus, 2 of the 8 tests were positive, but results of the following 3 *in vivo* test systems gave negative results:

Rat Hepatocyte DNA Repair Assay

Micronucleus Test (Mice)

Dominant Lethal Test (Mice)

Long-term carcinogenicity studies in mice and rats have been completed for ciprofloxacin. After daily oral doses of 750 mg/kg (mice) and 250 mg/kg (rats) were administered for up to 2 years, there was no evidence that ciprofloxacin had any carcinogenic or tumorigenic effects in these species. No long term studies of CIPRO<sup>®</sup> HC OTIC suspension have been performed to evaluate carcinogenic potential.

Fertility studies performed in rats at oral doses of ciprofloxacin up to 100 mg/kg/day revealed no evidence of impairment. This would be over 1000 times the maximum recommended clinical dose of ototopical ciprofloxacin based upon body surface area, assuming total absorption of ciprofloxacin from the ear of a patient treated with CIPRO<sup>®</sup> HC OTIC twice per day.

Long term studies have not been performed to evaluate the carcinogenic potential or the effect on fertility of topical hydrocortisone. Mutagenicity studies with hydrocortisone were negative.

#### Pregnancy

##### *Teratogenic Effects*

#### **Pregnancy Category C**

Reproduction studies have been performed in rats and mice using oral doses of up to 100 mg/kg and IV doses up to 30 mg/kg and have revealed no evidence of harm to the fetus as a result of ciprofloxacin. In rabbits, ciprofloxacin (30 and 100 mg/kg orally) produced gastrointestinal disturbances resulting in maternal weight loss and an increased incidence of abortion, but no teratogenicity was observed at either dose. After intravenous administration of doses up to 20 mg/kg, no maternal toxicity was produced in the rabbit, and no embryotoxicity or teratogenicity was observed.

Corticosteroids are generally teratogenic in laboratory animals when administered systemically at relatively low dosage levels. The more potent corticosteroids have been shown to be teratogenic after dermal application in laboratory animals.

Animal reproduction studies have not been conducted with CIPRO<sup>®</sup> HC OTIC. No adequate and well controlled studies have been performed in pregnant women. Caution should be exercised when CIPRO<sup>®</sup> HC OTIC is used by a pregnant woman.

#### Nursing Mothers

Ciprofloxacin is excreted in human milk with systemic use. It is not known whether ciprofloxacin is excreted in human milk following topical otic administration. Because of the potential for serious adverse reactions in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

**Pediatric use**

The safety and efficacy of CIPRO<sup>®</sup> HC OTIC have been established in pediatric patients 2 years and older (131 patients) in adequate and well-controlled clinical trials. Although no data are available on patients less than age 2 years, there are no known safety concerns or differences in the disease process in this population which would preclude use of this product in patients one year and older. See DOSAGE AND ADMINISTRATION.

**ADVERSE REACTIONS**

In Phase 3 clinical trials, a total of 564 patients were treated with CIPRO<sup>®</sup> HC OTIC. Adverse events with at least remote relationship to treatment included headache (1.2%) and pruritus (0.4%). The following treatment-related adverse events were each reported in a single patient: migraine, hypesthesia, paresthesia, fungal dermatitis, cough, rash, urticaria, and alopecia.

**DOSAGE AND ADMINISTRATION**

SHAKE WELL IMMEDIATELY BEFORE USING.

For children (age 1 year and older) and adults, 3 drops of the suspension should be instilled into the affected ear twice daily for seven days. The suspension should be warmed by holding the bottle in the hand for 1-2 minutes to avoid the dizziness which may result from the instillation of a cold solution into the ear canal. The patient should lie with the affected ear upward and then the drops should be instilled. This position should be maintained for 30-60 seconds to facilitate penetration of the drops into the ear. Repeat, if necessary, for the opposite ear. Discard unused portion after therapy is completed.

**HOW SUPPLIED**

CIPRO<sup>®</sup> HC OTIC is supplied as a white to off-white opaque suspension in a 10 mL bottle with a dropper dispenser.

NDC 0065-8531-10

Store below 77° F (25° C). Avoid freezing. Protect from light.

U.S. Patent Nos. 4,670,444; 4,844,902; 5,843,930; 5,965,549.

CIPRO is a registered trademark of Bayer AG.

Licensed by Bayer AG

Rx Only

Mfd. by:

ALCON CUSÍ, S.A.

08320 El Masnou - Barcelona

SPAIN

6-13-930

**CIPRO**

ciprofloxacin hydrochloride, hydrocortisone and benzyl alcohol suspension

**Product Information**

<b>Product Type</b>	HUMAN PRESCRIPTION DRUG	<b>NDC Product Code (Source)</b>	0065-8531
<b>Route of Administration</b>	AURICULAR (OTIC)	<b>DEA Schedule</b>	

**INGREDIENTS**

Name (Active Moiety)	Type	Strength
ciprofloxacin hydrochloride (ciprofloxacin)	Active	2 MILLIGRAM In 1 MILLILITER
hydrocortisone (hydrocortisone)	Active	10 MILLIGRAM In 1 MILLILITER
benzyl alcohol (benzyl alcohol)	Active	9 MILLIGRAM In 1 MILLILITER
polyvinyl alcohol	Inactive	

sodium chloride	Inactive
sodium acetate	Inactive
glacial acetic acid	Inactive
phospholipon 90H	Inactive
polysorbate	Inactive
water	Inactive
sodium hydroxide or hydrochloric acid	Inactive
<b>Product Characteristics</b>	
<b>Color</b>	<b>Score</b>
<b>Shape</b>	<b>Size</b>
<b>Flavor</b>	<b>Imprint Code</b>
<b>Contains</b>	
<b>Packaging</b>	
<b># NDC</b>	<b>Package Description</b>
1 0065-8531-10	10 mL (MILLILITER) In 1 BOTTLE, GLASS
2 0065-8531-23	10 mL (MILLILITER) In 1 BOTTLE, GLASS
	<b>Multilevel Packaging</b>
	None
	None

Revised: 04/2007

Alcon



Your Search Terms: PATENT

Version 1 - Published Feb 24, 2010

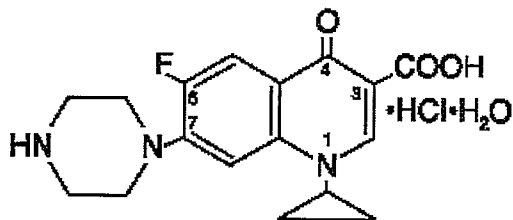
**CIPRO HC - ciprofloxacin hydrochloride , hydrocortisone and benzyl alcohol suspension**  
Stat Rx USA

**Cipro (ciprofloxacin hydrochloride, hydrocortisone and benzyl alcohol) Suspension**

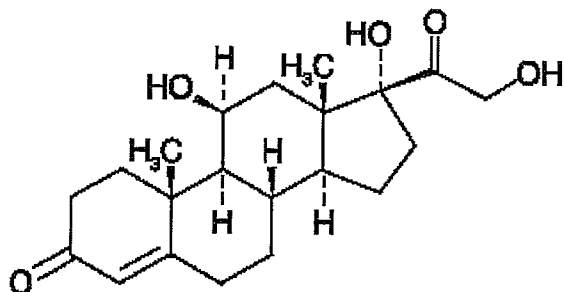
**DESCRIPTION**

CIPRO<sup>®</sup> HC OTIC (ciprofloxacin hydrochloride and hydrocortisone otic suspension) contains the synthetic broad spectrum antibacterial agent, ciprofloxacin hydrochloride, combined with the anti-inflammatory corticosteroid, hydrocortisone, in a preserved, nonsterile suspension for otic use. Each mL of CIPRO<sup>®</sup> HC OTIC contains ciprofloxacin hydrochloride (equivalent to 2 mg ciprofloxacin), 10 mg hydrocortisone, and 9 mg benzyl alcohol as a preservative. The inactive ingredients are polyvinyl alcohol, sodium chloride, sodium acetate, glacial acetic acid, phospholipon 90H (modified lecithin), polysorbate, and purified water. Sodium hydroxide or hydrochloric acid may be added for adjustment of pH.

Ciprofloxacin, a fluoroquinolone, is available as the monohydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. Its empirical formula is  $C_{17}H_{18}FN_3O_3 \cdot HCl \cdot H_2O$  and its chemical structure is as follows:



Hydrocortisone, pregn-4-ene-3, 20-dione, 11, 17, 21-trihydroxy-(11 $\beta$ )-, is an anti-inflammatory corticosteroid. Its empirical formula is  $C_{21}H_{30}O_5$  and its chemical structure is:



**CLINICAL PHARMACOLOGY**

The plasma concentrations of ciprofloxacin were not measured following three drops of otic suspension administration because the systemic exposure to ciprofloxacin is expected to be below the limit of quantitation of the assay (0.05 µg/mL).

Similarly, the predicted C<sub>max</sub> of hydrocortisone is within the range of endogenous hydrocortisone concentration (0-150 ng/mL), and therefore can not be differentiated from the endogenous cortisol.

Preclinical studies have shown that CIPRO<sup>®</sup> HC OTIC was not toxic to the guinea pig cochlea when administered intratympanically twice daily for 30 days and was only weakly irritating to rabbit skin upon repeated exposure.

Hydrocortisone has been added to aid in the resolution of the inflammatory response accompanying bacterial infection.

#### Microbiology

Ciprofloxacin has in vitro activity against a wide range of gram-positive and gram-negative microorganisms. The bactericidal action of ciprofloxacin results from interference with the enzyme, DNA gyrase, which is needed for the synthesis of bacterial DNA. Cross-resistance has been observed between ciprofloxacin and other fluoroquinolones. There is generally no cross-resistance between ciprofloxacin and other classes of antibacterial agents such as beta-lactams or aminoglycosides.

Ciprofloxacin has been shown to be active against most strains of the following microorganisms, both in vitro and in clinical infections of acute otitis externa as described in the **INDICATIONS AND USAGE** section:

#### **Aerobic gram-positive microorganism**

*Staphylococcus aureus*

#### **Aerobic gram-negative microorganisms**

*Proteus mirabilis*

*Pseudomonas aeruginosa*

#### **INDICATIONS AND USAGE**

CIPRO<sup>®</sup> HC OTIC is indicated for the treatment of acute otitis externa in adult and pediatric patients, one year and older, due to susceptible strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Proteus mirabilis*.

#### **CONTRAINDICATIONS**

CIPRO<sup>®</sup> HC OTIC is contraindicated in persons with a history of hypersensitivity to hydrocortisone, ciprofloxacin or any member of the quinolone class of antimicrobial agents. This nonsterile product should not be used if the tympanic membrane is perforated. Use of this product is contraindicated in viral infections of the external canal including varicella and herpes simplex infections.

#### **WARNINGS**

#### **NOT FOR OPHTHALMIC USE. NOT FOR INJECTION.**

CIPRO<sup>®</sup> HC OTIC should be discontinued at the first appearance of a skin rash or any other sign of hypersensitivity. Serious and occasionally fatal hypersensitivity (anaphylactic) reactions, some following the first dose, have been reported in patients receiving systemic quinolones. Serious acute hypersensitivity reactions may require immediate emergency treatment.

#### **PRECAUTIONS**

##### **GENERAL**

As with other antibiotic preparations, use of this product may result in overgrowth of nonsusceptible organisms, including fungi. If the infection is not improved after one week of therapy, cultures should be obtained to guide further treatment.

#### **Information for Patients**

If rash or allergic reaction occurs, discontinue use immediately and contact your physician.

Do not use in the eyes.

Avoid contaminating the dropper with material from the ear, fingers, or other sources.  
Protect from light.  
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Discard unused portion after therapy is completed.

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Eight *in vitro* mutagenicity tests have been conducted with ciprofloxacin, and the test results are listed below:

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Thus, 2 of the 8 tests were positive, but results of the following 3 *in vivo* test systems gave negative results:

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Dominant Lethal Test (Mice)

Long-term carcinogenicity studies in mice and rats have been completed for ciprofloxacin. After daily oral doses of 750 mg/kg (mice) and 250 mg/kg (rats) were administered for up to 2 years, there was no evidence that ciprofloxacin had any carcinogenic or tumorigenic effects in these species. No long term studies of CIPRO<sup>®</sup> HC OTIC suspension have been performed to evaluate carcinogenic potential.

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Teratogenic Effects

Pregnancy Category C

Reproduction studies have been performed in rats and mice using oral doses of up to 100 mg/kg and IV doses up to 30 mg/kg and have revealed no evidence of harm to the fetus as a result of ciprofloxacin. In rabbits, ciprofloxacin (30 and 100 mg/kg orally) produced gastrointestinal disturbances resulting in maternal weight loss and an increased incidence of abortion, but no teratogenicity was observed at either dose. After intravenous administration of doses up to 20 mg/kg, no maternal toxicity was produced in the rabbit, and no embryotoxicity or teratogenicity was observed.

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Animal reproduction studies have not been conducted with CIPRO<sup>®</sup> HC OTIC. No adequate and well controlled studies have been performed in pregnant women. Caution should be exercised when CIPRO<sup>®</sup> HC OTIC is used by a pregnant woman.

### **Nursing Mothers**

Ciprofloxacin is excreted in human milk with systemic use. It is not known whether ciprofloxacin is excreted in human milk following topical otic administration. Because of the potential for serious adverse reactions in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

**Pediatric use**

The safety and efficacy of CIPRO® HC OTIC have been established in pediatric patients 2 years and older (131 patients) in adequate and well-controlled clinical trials. Although no data are available on patients less than age 2 years, there are no known safety concerns or differences in the disease process in this population which would preclude use of this product in patients one year and older. See DOSAGE AND ADMINISTRATION.

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**DOSAGE AND ADMINISTRATION**

SHAKE WELL IMMEDIATELY BEFORE USING.

For children (age 1 year and older) and adults, 3 drops of the suspension should be instilled into the affected ear twice daily for seven days. The suspension should be warmed by holding the bottle in the hand for 1-2 minutes to avoid the dizziness which may result from the instillation of a cold solution into the ear canal. The patient should lie with the affected ear upward and then the drops should be instilled. This position should be maintained for 30-60 seconds to facilitate penetration of the drops into the ear. Repeat, if necessary, for the opposite ear. Discard unused portion after therapy is completed.

**HOW SUPPLIED**

CIPRO® HC OTIC is supplied as a white to off-white opaque suspension in a 10 mL bottle with a dropper dispenser.

NDC 0065-8531-10

Store below 77° F (25° C). Avoid freezing. Protect from light.

U.S. Patent Nos. 4,670,444; 4,844,902; 5,843,930; 5,965,549.

CIPRO is a registered trademark of Bayer AG.

Licensed by Bayer AG

Rx Only

Mfd. by:

ALCON CUSÍ, S.A.

08320 El Masnou - Barcelona

SPAIN

6-13-930

**CIPRO HC OTIC SUSP Package Label**

Packaged and distributed by: **Stat Rx USA** Gainesville, GA 30501

**Cipro HC Otic Susp**  
0.2% / 1% 10 mL

Generic For:

**NDC 16590-053-10** Prod# 053-10 Lot# SAMPLE

Each mL Contains: Ciprofloxacin Hcl. 0.2% / Hydrocortisone 1%

Mfg By: 3M Health Care  
St. Paul, MN 55144 NDC 0065-8531-10

Mfg Lot: SAMPLE  
Discard After: 01/12 MD 7/17/2009 9259997

**RX ONLY-KEEP OUT OF REACH OF CHILDREN**

Caution: Federal law prohibits transferring this drug to any person other than the patient for whom it was prescribed.

**SHAKE WELL & FOR EARS**



<b>CIPRO HC</b> ciprofloxacin hydrochloride suspension			
<b>Product Information</b>			
Product Type	HUMAN PRESCRIPTION DRUG	NDC Product Code (Source)	16590-053 (0065-8531)
Route of Administration	AURICULAR (OTIC)	DEA Schedule	
<b>Active Ingredient/Active Moiety</b>			
Ingredient Name	Basis of Strength	Strength	
ciprofloxacin hydrochloride (ciprofloxacin)	ciprofloxacin hydrochloride	2 mg in 1 mL	
hydrocortisone (hydrocortisone)	hydrocortisone	10 mg in 1 mL	
benzyl alcohol (benzyl alcohol)	benzyl alcohol	9 mg in 1 mL	
<b>Inactive Ingredients</b>			
Ingredient Name	Strength		
No Inactive Ingredients Found			
<b>Product Characteristics</b>			
Color	Score		
Shape	Size		
Flavor	Imprint Code		
Contains			
<b>Packaging</b>			
# NDC	Package Description	Multilevel Packaging	
1 16590-053-10	10 mL In 1 BOTTLE, GLASS	None	

**Marketing Information**

Marketing Category	Application Number or Monograph Citation	Marketing Start Date	Marketing End Date
NDA	NDA020805	02/10/1998	

**Labeler** - Stat Rx USA (786036330)

Revised: 10/2009

Stat Rx USA

EXHIBIT D  
PATENT 4,844,902

# United States Patent [19] Grohe

[11] Patent Number: 4,844,902

[45] Date of Patent: Jul. 4, 1989

[54] TOPICALLY APPLICABLE  
FORMULATIONS OF GYRASE INHIBITORS  
IN COMBINATION WITH  
CORTICOSTEROIDS

[75] Inventor: Klaus Grohe, Odenthal, Fed. Rep. of Germany

[73] Assignee: Bayer Aktiengesellschaft,  
Leverkusen, Fed. Rep. of Germany

[21] Appl. No.: 154,835

[22] Filed: Feb. 11, 1988

[30] Foreign Application Priority Data

Feb. 17, 1987 [DE] Fed. Rep. of Germany ..... 3704907

[51] Int. Cl.<sup>4</sup> ..... A61F 13/00

[52] U.S. Cl. .... 424/449; 424/447

[58] Field of Search ..... 424/449

[56] References Cited

## U.S. PATENT DOCUMENTS

4,659,603 4/1987 Groke et al. .... 514/254

4,681,876 7/1987 Marpla et al. .... 514/182

## OTHER PUBLICATIONS

BE-A- 829 197 (L. Grosjean).

GB-A-2 116 425 (Rhom Pharma).

Embase 86048074, 0150500902110.

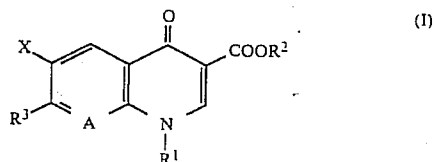
Primary Examiner—Ellis P. Robinson

Assistant Examiner—Leon R. Horne

Attorney, Agent, or Firm—Sprung Horn Kramer & Woods

## [57] ABSTRACT

Topically applicable formulations comprising known ciprofloxacin-type antibacterials of the formula



in which

A is N or C-R<sup>9</sup>,

and corticosteroids are especially effective in therapy, particularly in the oral cavity. The formulations can be used in the form of plasters, gels, suspensions, emulsions and solutions.

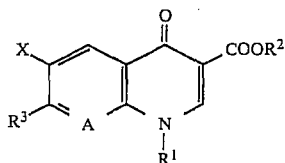
11 Claims, No Drawings

4,844,902

1

# TOPICALLY APPLICABLE FORMULATIONS OF GYRASE INHIBITORS IN COMBINATION WITH CORTICOSTEROIDS

The invention relates to topically applicable formulations which contain, as active compounds, antibacterially active compounds which belong to the group of gyrase inhibitors and have the general formula

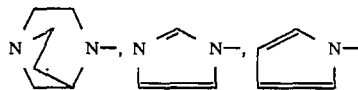
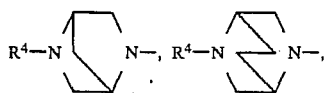
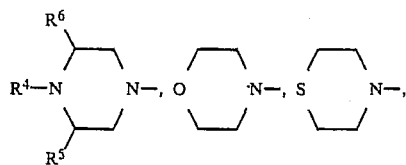
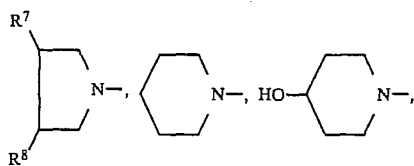


in which

R<sup>1</sup> represents methyl, ethyl, propyl, isopropyl, cyclopropyl, vinyl, 2-hydroxyethyl, 2-fluoroethyl, methoxy, amino, methylamino, dimethylamino, ethylamino, phenyl, 4-fluorophenyl and 2,4-difluorophenyl,

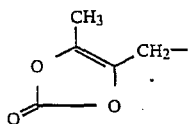
R<sup>2</sup> represents hydrogen, alkyl with 1 to 4 carbon atoms or (5-methyl-2-oxo-1,3-dioxol-4-yl)-methyl,

R<sup>3</sup> represents methyl or a cyclic amino group, such as



wherein

R<sup>4</sup> represents hydrogen, alkyl with 1 to 4 carbon atoms, 2-hydroxyethyl, allyl, propargyl, 2-oxo-propyl, 3-oxobutyl, phenacyl, formyl, CFCL<sub>2</sub>-S-CFCL<sub>2</sub>-SO<sub>2</sub>-, CH<sub>3</sub>O-CO-SO-, benzyl, 4-aminobenzyl or



2

R<sup>5</sup> represents hydrogen or methyl,

R<sup>6</sup> represents hydrogen, alkyl with 1 to 4 carbon atoms, phenyl or benzyloxymethyl,

R<sup>7</sup> represents hydrogen, amino, methylamino, ethylamino, aminomethyl, methylaminomethyl, ethylaminomethyl, dimethylaminomethyl, hydroxyl or hydroxymethyl and

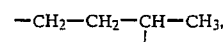
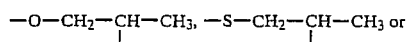
R<sup>8</sup> represents hydrogen, methyl, ethyl or chlorine,

X represents hydrogen, fluorine, chlorine or nitro and A represents N or C-R<sup>9</sup>,

(I) wherein

R<sup>9</sup> represents hydrogen, halogen, such as fluorine or chlorine, methyl or nitro, or

A, together with R<sup>1</sup>, can also form a bridge with the structure



and a corticosteroid or several corticosteroids.

The gyrase inhibitors can be used in the topically applicable formulations as such or as a salt with an acid or base. Use as a prodrug, for example of esters, is also possible.

The gyrase inhibitors are applied topically in combination with corticosteroids in the form of the topically applicable formulations for the treatment or prophylaxis of infections, diseases and injuries to the skin, including burns. Treatment or prophylaxis of deeplying or systemic infections is also possible by topical application or gyrase inhibitors.

The topically applicable formulations according to the invention contain 0.05 to 30% preferably 0.05 to 20% by weight of active compound of the formula (I) and corticosteroids.

The topically applicable formulations according to the invention particularly preferably contain 0.1 to 5% by weight of active compound of the formula (I) and topically.

The formulations mentioned contain, in particular, ciprofloxacin, norfloxacin, pefloxacin, amifloxacin, pirofloxacin, ofloxacin and/or enoxacin.

The corticosteroid active compounds which are used in the formulations according to the invention are known. Such active compounds are described in detail, for example, in Miller, Zunro, Dengs 19, 119-134 (1980) and Wolfe, Bayer's Medicinal Chemistry 3 4A Ed., John Wiley and Bus, New York, N.Y. pages 1273-1316, 917-1309 (1981).

Other corticosteroid active compounds which are suitable as constituents of the formulations according to the invention are described in: European Pat. No. 0,036,138, European Pat. No. 0,129,283, European Pat. No. 0,098,566, European Pat. No. 0,173,478, European Pat. No. 0,136,586, European Pat. No. 0,098,568, European Pat. No. 0,095,894, European Pat. No. 0,078,235, European Pat. No. 0,023,713, German Pat. No. 3,227,312, German Pat. No. 3,243,482, German Pat. No. 3,401,680, German Pat. No. 3,400,188, U.S. Pat. No. 4,257,969 and U.S. Pat. No. 4,343,798.

Preferred corticosteroids are hydroxytriamcinol-one,  $\alpha$ -methyl-dexamethasone,  $\beta$ -methyl-betamethasone, beclomethasone,  $\alpha$ -propionate, betamethasone bonzo-



4,844,902

3

ate, betamethasone dipropionate, betamethasone valerate, clobetasol valerate, desonide, desoxymethasone, dexamethasone, diflorasone diacetate, diflucortolone valerate, flurandrenolone, flucinolone acetonide, flumethasone pivalate, flucinolone acetonide, fluocinonide, fluocortin butyl ester, fluocortolone, fluprednidene acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, diflorasone diacetate, flurandrenolone acetonide, medrysone, amcinafal, amcinafide, betamethasone and esters thereof, chloroprednisone, clocortolone, clescinalone, dichlorisone, difluprednate, flucoronide, flunisolid, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone and beclomethasone dipropionate.

Mixtures of the so-called corticosteroids are also possible constituents of the formulations according to the invention.

Examples, in preferred % by weight, of the corticosteroids which can be used according to the invention are, in particular:

- A beclomethasone dipropionate 0.5%
- B clobetasol propionate 0.05%
- C diflucortolone valerate 0.3%
- D fluocinolone acetonide 0.2%
- E beclomethasone dipropionate 0.025%
- F betamethasone benzoate 0.025%
- G betamethasone dipropionate 0.05%
- H betamethasone valerate 0.1%
- I desonide 0.05%
- J desoxymethasone 0.25%
- K diflorasone diacetate 0.05%
- L diflucortolone valerate 0.1%
- M flucinolone acetonide 0.025%
- N fluocinolone acetonide 0.025%
- O fluocinonide 0.05%
- P fluocortolone 0.5%
- Q fluprednidene (fluprednylidene) acetate 0.1%
- R flurandrenolone 0.05%
- S halcinonide 0.1%
- T hydrocortisone butyrate 0.1%
- X triamcinolone acetonide 0.1%
- Y clobetasone butyrate 0.05%
- Z flumethasone pivalate 0.02%
- A<sup>1</sup> fluocinolone acetonide 0.01%
- B<sup>1</sup> fluocortine butyl ester 0.75%
- C<sup>1</sup> fluocortolone 0.2%
- D<sup>1</sup> flurandrenolone 0.0125%-0.025%
- E<sup>1</sup> hydrocortisone (urea) 1%
- F<sup>1</sup> dexamethasone 0.01%
- G<sup>1</sup> hydrocortisone (alcohol or acetate) 0.1%-1%
- H<sup>1</sup> methylprednisolone 0.25%

Corticosteroids from the group consisting of triamcinolone acetonide, hydrocortisone acetate, betamethasone valerate, fluocinolone acetonide, flupamesone and mixtures of these compounds are particularly preferred.

Other preferred active compounds are desoxycorticosterone, fludrocortisone, hydrocortisone, betamethasone, cortisone, dexamethasone, prednisolone, prednisone, methylprednisolone, paramethasone, triamcinolone and mixtures of these compounds.

The formulations according to the invention contain the corticosteroids in amounts of 0.01 to 10% by weight, preferably 0.02 to 5% by weight and particularly preferably 0.05 to 5% by weight.

4

#### A. General section

The topical formulations of the invention include solutions, sprays, lotions, gels, ointments, creams, powders, dusting powder sprays, pastes, suspensions, emulsions, foams and sticks containing the active compound of the formula I, and if appropriate also several active compounds.

The present compounds of the formula I can also be applied topically in the form of plasters, spray plasters, occlusive dressings, compresses and controlled release systems. These formulations can contain the active compounds in dissolved or suspended form.

Ointments contain, as the base, hydrocarbon gels, lipogels, absorption bases, water-in-oil ointment bases, mixed emulsions or polyethylene glycols.

Creams contain oil-in-water bases.

Pastes contain, in addition to an ointment or cream base, high amounts of pulverulent constituents, such as zinc oxide, talc, starch or titanium dioxide.

Gels contain solvents, such as water, ethanol, isopropanol or propylene glycol, and are prepared using gelling agents, such as cellulose ethers, alginates, polyacrylates, bentonite, gelatine, tragacanth, polyvinylpyrrolidone or polyvinyl alcohol. It is also possible to use lipophilic gel bases or microemulsions.

Dusting powders contain pulverulent additives, such as starch, stearate, silicon dioxide, clay, magnesium carbonate, talc, cellulose, zinc oxide and, in particular, lactose.

Stabilizers, antioxidants, preservatives, humectants, regreasing agents, solvents or auxiliaries can be added to all the formulations to improve the penetration and efficacy.

Examples of agents which improve penetration are propylene glycol, polyethylene glycol, dimethylsulphoxide, decylmethylsulphoxide, azones, N-methylpyrrolidone, diethyltoluamide, ethanol, isopropyl myristate, isopropyl palmitate, oleic acid and its esters, medium-chain triglycerides, dimethyl isosorbitol, 2-octyldodecanol, branched fatty acids, benzyl alcohol, urea, salicylates and surfactants.

#### B. Adhesive topical formulations

Where animals are treated, it has proved advantageous to use adhesive topically applicable formulations and to implement the invention as illustrated by way of example below.

Topical formulations according to the invention which can be applied to both wet and dry animals are characterized, for example, in that they contain (a<sup>1</sup>) 0.1-20%, preferably 0.1-5%, of an active compound of the formula I, (a<sup>2</sup>) 0.01 to 10% of a corticosteroid, (b) 1-40%, preferably 1-20%, of a water-soluble gel-or lacquer-forming polymer, (c) 40-98%, preferably 60-90%, of an organic water-miscible solvent which evaporates faster than water and in which the polymer does not dissolve, and (d) 0.1-10% of various additives, for example plasticizers, suspending auxiliaries, antioxidants, spreading agents, dyestuffs and the like.

To prepare the formulations, polymers which are known per se or salts thereof are suspended in a solvent in which they are not soluble. On the other hand, the polymers swell in water to form a gel. The active compound is either suspended or dissolved in the solvent. The solvent must be water-miscible and be able to evaporate faster than water. The customary formulation auxiliaries can be added to the suspension in order to

5

4,844,902

guarantee a suspension which can easily be shaken up or is homogeneous. It may also be desirable to add a plasticizer so that the film which forms is later kept elastic.

If such a suspension is poured or sprayed onto a wet animal, as the solvent evaporates the polymer swells to form a gel which dries out to a lacquer or film layer and thereby incorporates the active compound. This layer remains stuck to the coat of hair or skin for a long time and is washed off only slowly - gradually - by showers of rain or a dipping bath.

The suspension is diluted with water in approximately equal proportions. It then still has a low viscosity and the polymer has not yet swollen, so that it can be applied effortlessly with the usual equipment. Here also, a gel and later a film forms after the solvent has evaporated, as already described above.

Possible gel- and film-forming agents are all the macromolecular compounds which do not dissolve in the water-miscible organic solvent and, after mixing with water, swell to form a gel which gives a type of film after drying.

Following a classification of macromolecular auxiliaries such as is described, for example, by Keipert et al. in *Die Pharmazie* 28, 145-183 (1973), above all ionic macromolecules in their salt form are used. These are, inter alia, sodium carboxymethylcellulose, polyacrylic acid, polymethacrylic acid and salts thereof, sodium amylopectin semiglycolate, alginic acid and propylene glycol alginate as the sodium salt, gum arabic, xanthan gum and guar gum.

Amphoteric macromolecules, such as protein derivatives, for example gelatine, are just as suitable as non-ionic polymers, for example methylcellulose, other cellulose derivatives and soluble starches, which meet the above requirements.

Suitable solvents are all the water-miscible liquids which do not dissolve the macromolecule and evaporate faster than water.

Examples of possible solvents are alkanols, such as ethanol and isopropyl alcohol; ketones, such as acetone and methyl ethyl ketone, and glycol ethers, such as ethylene glycol monomethyl ether or monoethyl ether.

One or more solvents can be used in the preparation of the formulations according to the invention of the type described above.

Other auxiliaries which are suitable for such formulations are:

(a) Substances which can stabilize the suspension, for example colloidal silicic acid, montmorillonites and the like,

(b) Surfactants (including emulsifiers and wetting agents), for example

1. anionic surfactants, such as Na lauryl-sulphate, fatty alcohol ether-sulphates and mono/dialkyl polyglycol ether-orthophosphoric acid ester mono-ethanolamine salts;
2. cationic surfactants, such as cetyltrimethylammonium chloride;
3. amphoteric surfactants, such as di-Na N-lauryl-B-iminodipropionate or lecithin; and
4. non-ionic surfactants, for example polyoxyethylated castor oil, polyoxyethylated sorbitan monooleate, sorbitan monostearate, cetyl alcohol, glycerol monostearate, polyoxyethylene stearate and alkylphenol polyglycol ethers,

(c) Stabilizers for preventing the chemical degradation which occurs with some active compounds, such as

6

antioxidants, for example tocopherols and butylhydroxyanisole, and

(d) Plasticizers for the elasticity of the film-forming agents, for example glycerol and propylene glycol.

All the formulations customary in dermatology and cosmetics are preferably suitable as use forms for application of the topical formulations according to the invention for combating bacterial diseases in humans. Formulations which may be mentioned in particular are those which, after application to the skin, are not immediately washed off on contact with water, that is to say those which contain film-forming or water-repellent additives, such as the formulations already mentioned above. Formulations of this type are, for example, solutions, sprays, lotions and ointments (here both emulsion ointments and suspension ointments and sticks analogous to insect repellent sticks).

#### C. Liquid formulations with spreading agents

Spreading oils can also be added to the formulations according to the invention, if these are in liquid form, for better distribution on surfaces, in particular on the skin.

Spreading oils are understood as those oily liquids which are distributed particularly easily on the skin. They are known as such in cosmetics. According to a proposal by R. Reymer, *Pharm. Ind.* 32, 577 (1970), they can be characterized, for example, by their surface tension in respect of air, which should accordingly be less than 30 dynes/cm.

The following substances are particularly suitable spreading agents:

Silicone oil of varying viscosity

Fatty acid esters, such as ethyl stearate, di-n-butyl adipate, hexyl laurate and dipropylene glycol pelargonate, esters of a branched fatty acid of medium chain length with saturated C<sub>16</sub>-C<sub>18</sub> fatty alcohols, isopropyl myristate, isopropyl palmitate, caprylic/capric acid esters of saturated fatty alcohols of C<sub>12</sub>-C<sub>18</sub> chain length, isopropyl stearate, oleyl oleate, decyl oleate, ethyl oleate, ethyl lactate, waxy fatty acid esters, such as synthetic duck uropygial gland fat, dibutyl phthalate, diisopropyl adipate, ester mixtures related to the latter and the like.

Triglycerides, such as caprylic/capric acid triglyceride, triglyceride mixtures with vegetable fatty acids of C<sub>8</sub>-C<sub>12</sub> chain length or other specifically selected naturally occurring fatty acids, partial glyceride mixtures of saturated and unsaturated fatty acids which optionally also contain hydroxyl groups, monodiglycerides of the C<sub>8</sub>/C<sub>10</sub>-fatty acids and others.

Fatty alcohols, such as isotridecyl alcohol, 2-octyldodecanol, cetylstearyl alcohol and oleyl alcohol.

Fatty acids, such as oleic acid.

Particularly suitable spreading oils are the following: isopropyl myristate, isopropyl palmitate, caprylic/capric acid esters of saturated fatty alcohols of C<sub>18</sub>-C<sub>18</sub> chain length and waxy fatty acid esters, such as synthetic duck uropygial gland fat.

#### D. Plasters

Other topically applicable formulations of the present invention are medicinal plasters for release of the active compounds of the formula I to the skin over a prolonged period of time.

The invention accordingly also relates to medicinal plasters for administration of an active compound of the formula I to the skin, which contain a top layer consist-

4,844,902

7

ing of a longitudinally-transversely elastic, preferably textile sheet-like structure, in particular a knitted fabric or mesh fabric, impregnated or coated with a polymer, a reservoir layer and a pull-off protective layer, the reservoir layer containing a polymer consisting of polyisobutylene and/or copolymers thereof, an entraining agent and a resin.

Polymers in the sense of this section of the invention are preferably understood as polyisobutylene and/or copolymers thereof.

Polyisobutylenes in the sense of the invention are understood as polyisobutylenes which, due to their preparation, have a molecular weight distribution  $M_w/M_n$  of 1.5 to 3.5, preferably 2.0 to 3.0, and a viscosity-average molecular weight—again as a result of their preparation—of 30,000 to 4,000,000 g/mol. The viscosity-average molecular weight of the polyisobutylenes to be employed according to the invention is preferably 50,000 to 1,000,000 g/mol, particularly preferably 80,000 to 500,000 g/mol. The viscosity-average molecular weight can be determined in a known manner as described in the Polymer Handbook, J. Brandrup and F. H. Immergut, Wiley & Sons, N.Y., 1975, Chapter IV, page 35.

These polyisobutylenes have been known for a long time and can be prepared, for example, according to U.S. Patent No. 2,203,873 or according to German Patent No. 704,038 with acid catalysts.

Copolymers of isobutylene in the sense of the invention are those of isobutylene with 0.5 to 5 mol % of conjugated diolefins, preferably those with 4 to 6 C atoms, such as, for example, buta-1,3-diene, piperylene and 2,3-dimethylbutadiene, and particularly preferably with isoprene, the molecular weights of which can be 30,000 to 200,000 g/mol. These isobutene copolymers are also known. Polyisobutylene homopolymers with a viscosity-average molecular weight of 80,000 to 500,000 are particularly preferably employed.

Entraining agents in the context of the invention are to be understood as oils, fatty acid esters, triglycerides, alcohols and/or fatty acids.

Oils in the sense of that part of the invention concerning medicinal plasters are to be understood as high-boiling aliphatic, araliphatic and/or aromatic hydrocarbons, preferably paraffin oil, purcellin oil, perhydrosqualene and solutions of microcrystalline waxes in oils, and mineral oils, preferably oils with a boiling range of between 150° C. and 400° C.; and furthermore unsaturated hydrocarbons with at least 16 C atoms, such as, for example, oligomers of monoolefins, such as tetraisobutylene, pentaisobutylene or hexaisobutylene, or liquid polymers of diene(monoene) (co)polymers. Examples of liquid polymers of conjugated dienes are those of butadiene, isoprene, 1,3-pentadiene and 2,3-dimethylbutadiene, copolymers of various dienes and liquid copolymers of a conjugated diolefin and small amounts of monoolefins, such as, for example, but-1-ene, isobutene, hex-1-ene, oct-1-ene or styrene, with a molecular weight of 400 to 6,000, preferably 800 to 3,000, iodine numbers of 200 to 500 and viscosities of 100 to 10,000 cP at 50° C.

Liquid polybutadiene polymers which are at least 90% 1,4-linked and have a content of cis double bonds of more than 60% and molecular weights of 1,000 to 4,000 are particularly preferred.

Oils are also understood as being silicone oils of varying viscosity, preferably with average molecular

8

weights of 312 to 15,000, particularly preferably polydimethylsiloxanes.

Fatty acid esters are to be understood as those which contain at least 12 C atoms, preferably 15 to 46 C atoms and particularly preferably 16 to 36 C atoms.

Amongst these, there are to be understood in particular: ethyl stearate, hexyl laurate, dipropylene glycol pelargonate, cetyl palmitate, isopropyl myristate, isopropyl palmitate, caprylic/capric acid esters of saturated fatty alcohols of C<sub>12</sub>–C<sub>18</sub> chain length, isopropyl stearate, oleyl oleate, decyl oleate and synthetic duck uropygial gland fat, and in particular in each case individually or as a mixture.

Triglycerides are understood as being pure or mixed esters of glycerol with fatty acids of C<sub>8</sub>–C<sub>18</sub> chain length, preferably caprylic and/or capric acid triglycerides.

Fatty acids are understood as being saturated or unsaturated fatty acids, preferably those with 12 to 24 C atoms, individually or as mixtures with one another, and particularly preferably oleic acid.

Oils in the sense of the invention are furthermore understood as being: sweet almond oil, avocado oil, sesame oil, castor oil, olive oil, grapeseed oil, clove oil, groundnut oil, corn oil, hazelnut oil, jojoba oil, carthamus oil and wheatgerm oil, in each case individually or as a mixture.

Resins in the sense of that part of the invention which affects plasters are understood as being rosin, dehydrogenated rosin, glycerol esters of dehydrogenated rosin, glycerol esters of rosin gum, hydrogenated rosin, glycerol esters of hydrogenated rosin, pentaerythritol esters of hydrogenated rosin, methyl esters of hydrogenated rosin, polymerized rosin, glycerol esters of polymerized rosin, terpene resins, coumarone/indene resins, hydrogenated petroleum resins, such as maleic anhydride-modified rosin and rosin derivatives, C<sub>5</sub>-petroleum resins and half-esters of styrene/maleic acid copolymers, individually or as in a mixture with one another. Polyterpene resins of alpha- or beta-pinene or modified glycerol esters of rosin are particularly preferred. Depending on the properties required in respect of tackiness and adhesion to the area to which the resulting plaster is to be applied, these resins can be used either by themselves or in combination with one another.

The active compounds of the formula 2 can be incorporated into the reservoir layer in the plasters of the invention in an amount of 1 to 30% by weight, preferably 2 to 20% by weight. The percentages by weight relate to the total reservoir.

Active substances or cooling or fragrant substances, preferably methyl salicylate, glycol salicylate, salicylic acid, menthol, peppermint oil, camphor, thymol, acrinol, scopolia extract, chlorpheniramine maleate, benzyl nicotinate, capsicum extract, nonylvanillylamine and capsaicin, can additionally also be added to the active substances of the formula I.

If necessary, additives and fillers, for example anti-ageing agents, antioxidants and reinforcing fillers, can be added to the plasters according to the invention.

Longitudinally and transversely elastic knitted fabrics and mesh fabrics have been used as the covering layer for the plasters according to the invention (see, for example, Koch-Satlow, Grosses Textillexikon (Large Textile Encyclopaedia), Deutsche Verlagsanstalt Stuttgart 1965).

Knitted fabrics and mesh fabrics are accordingly textile sheet-like structures which are produced from



one or more thread systems on knitting machines by forming stitches. A distinction is made between two categories: weft knitted and mesh fabric (main feature: threads run in the transverse direction, analogous to the filling direction of woven fabrics) and warp knitted fabric (main feature: threads run in the longitudinal direction, analogous to the warp direction of woven fabrics).

The separation of terms into knitted fabric and mesh fabric usual in specialist terminology relates to the production process. In knitting, the stitches of one row of stitches are formed (sloughed) at the same time, whereas in meshing one stitch is formed after the other. However, there are exceptions to the assignment of the terms. From the point of view of binding, there is no difference between weft knitted fabrics and mesh fabrics.

In contrast to woven fabrics, knitted fabrics and to woven fabrics, knitted fabrics and mesh fabrics have a high extension and elasticity, especially in the transverse direction; moreover, because of the stitch structure, they have a large pore volume, which promotes permeability to air and thermal insulation. These and other properties can be varied substantially by their formation and also by the choice of fibre and yarn.

The knitted fabrics and mesh fabrics used according to the invention preferably have a stretch character. The customary methods of textile technology are used to achieve this stretch character (see Koch-Satlow, page 441), or elastomer fibres or elastomer yarns are used directly when choosing the base materials for the knitted fabric and mesh fabric.

As well as knitted fabrics and mesh fabrics, textile sheet-like structures with a stretch character can in general be used as the covering layer for the plasters according to the invention, that is to say all three-dimensional structures of natural and synthetic textile fibers, such as plaited materials, non-wovens or felts, are suitable as the top layer.

Base materials which can be used for the top layer are, inter alia, fibers and filaments of polyamide, polyester, polyurethane, polyamide-polyurethane, cotton, viscose staple and animal wool.

The textile top layer of the plasters according to the invention is impregnated or coated. The customary techniques and materials are used for coating and impregnation (see also Koch-Satlow, pages 157 to 159 and page 616 et seq.).

The top layer is preferably impregnated or coated with polyisobutylene. The molecular weight of the polyisobutylene here is preferably <1,000,000 g/mol (viscosity-average).

Preferred possible coating and impregnating materials are the polyisobutylenes which are also contained in the reservoir layer, but they have higher molecular weights and are not tacky.

The pull-off film of the plasters according to the invention can consist of occlusive, flexible or non-flexible materials, such as polyethylene, polypropylene, polyethylene, terephthalate, nylon and other known films. Metal foils, such as aluminum foil, by themselves or laminated with polymers, can also be used as the pull-off film. Multi-layer films, such as laminates of polyethylene with polyester PE terephthalate and vapor-deposited with aluminum, can also be used. Other pull-off films are, inter alia, polyesters treated with silicone, polyethylene terephthalate with terminal sili-

cone groups, treated paper, silicone-treated paper, paper coated with polyethylene and the like.

#### E. Gels

The invention preferably also relates to topically applicable formulations in the form of gels. Gels here are understood as being disperse "solid/liquid" systems in which the disperse phase is no longer freely mobile.

Topical formulations of the active compounds of the formula I which are in the form of gels are suitable for the treatment of bacterial infections of, preferably, body cavities, in particular the oral cavity. The depot action, good adhesion properties and higher bioavailability of the active substances permit short-term therapy.

In order to shorten the duration of the therapy, a certain depot action and a higher bioavailability of the active compounds is required. The formulations according to the invention in gel form are particularly suitable for this. If a shortening in the duration of therapy is to be achieved without a further increase in the active compound concentration, optimum bioavailability of the active substance must be ensured.

In the region of the oral mucosa in particular, formulations which on the one hand have an adequate adhesion after application to the oral mucosa and on the other hand can release a sufficient amount of the active compound contained in the formulation, even in solution in the saliva, are therefore required.

It has been found that those formulations of the active compounds of the formula I in combination with corticosteroids which contain a cellulose ether, in particular hydroxypropylcellulose, sodium alginate or propylene glycol alginate as the gel-forming agent and in addition the customary formulation auxiliaries facilitate optimum adhesion properties and optimum release of the active compound and therefore a shortened duration of therapy by achieving antibacterial concentrations of the active compound. This effect is achieved by the bioavailability if the active compounds contained in the formulations being increased by adhesive properties, so that the release of active compound in the saliva can be increased.

Active compounds which can be formulated in this manner are all the active compounds of the formula I, in particular ciprofloxacin and the other active compounds mentioned on page 3. They are preferably present in the gels according to the invention in amounts of 0.05 to 30% by weight, preferably 0.05 to 0.5% by weight and in particular 0.1 to 1% by weight. The amounts of corticosteroid correspond to the amounts stated above.

Possible gel-forming agents are those macromolecular compounds which can dissolve or swell both in water and in organic solvents. Cellulose ethers may be mentioned above all here, and 2.5 to 17.5% of these are required.

Following a classification of the macromolecular auxiliaries (Keipert et al., *Die Pharmazie* 28, 145-183 (1973)), above all ionic macromolecules in salt form are used. These are, inter alia, sodium carboxymethylcellulose, polyacrylic acid, polymethacrylic acid and salts thereof, sodium amylopectinsemiglycolate, alginic acid and propylene glycol alginate as the sodium salt, gum arabic and guar gum.

Amphoteric macromolecules, such as protein derivatives, for example gelatine, are just as suitable as non-ionic polymers, for example methylcellulose, hydroxy-

4,844,902

11

propylcellulose and soluble starches, which meet the above requirements.

Possible gel-forming agents, which also have a stabilizing action, are long-chain linear high molecular weight polysaccharides with a molecular weight of more than one million. 0.1 to 1.5% of such stabilizers is required.

Suitable solvents are water and also all water-miscible solvents. Examples of possible solvents are alkanols, such as ethanol and isopropyl alcohol, benzyl alcohol, propylene glycol and the like.

Those macromolecular compounds such as, for example, hydroxypropylcellulose (probable molecular weight 2,000,000) have been found to be particularly stable gel combination bases.

The gels according to the invention preferably contain 2.5 to 35.0% by weight of the spreading agents mentioned under C.

#### F. Dusting powders

Topically applicable formulations according to the invention in dusting powder form contain the active compounds of the formula I and corticosteroids, and preferably dusting powder bases, such as rice starch, corn starch, wheat starch, talc, Bolus alba and Bolus rubra, kieselguhr, Aerosil, magnesium oxide, magnesium carbonate, zinc oxide and titanium dioxide.

The following dusting powder raw materials can in general be used according to the invention: Aerosil, aluminum hydroxide, palmitate and stearate, amyllum non mucilaginosum, arrowroot, Avicel microcrystalline cellulose, barium sulphate, bolus, Cab-o-sil, calamine, calcium carbonicum praecipitatum, calcium silicate, stearate and sulphate, Calflo, dextrans, ekasil, flosilite, Glaxie Micron, gasil, kaolin, kieselguhr, silicic acid D 17, kirusol, Kronos titanium dioxide pigments, laminarin, luvokoll, magnesium carbonate, oxide, silicate, stearate and trisilicate, magnesol, marinco, metallic soaps, metasap, lactose, NAL, neosyl, oracid powder, ottalume, polyethylene, polyamide powder, polyester powder, Pontybond 2150, Powdertrol, Quso, rice starch, Santocel C, Sea sorb, soap fibroin, Sicol, Siflox, silica gel, Silin S 100, S-Micron Silica, Syloid, Talcum cetylalum, talc, Tego metallic soaps, Tiotal, Tioxide, titanium dioxide, titanium stearate, Veegum, Vinyon, Witcarb and zinc carbonate, myristate, oxide and stearate.

Starch, in particular rice starch and wheat starch, are preferably used as the dusting powder base for the active compounds of the formula I and the corticosteroids. Etherified starches are also preferably used.

Dusting powders which contain the active compounds are prepared with solid active compounds of the formula I and the corticosteroids by mixing the active compounds in finely divided form with the corresponding amount of dusting powder base from the point of view of mixing of powders.

They can also be introduced into aerosol compressed gas packs together with liquefied propellants and used as sprayable dusting powders. For these, about 8 to 9 parts by weight of propellant are to be used per part of dusting powder.

Liquid or semi-solid active compounds of the formula I and the corticosteroids are triturated intimately with a small amount of the base and the composition, which is then dried, is mixed with the remainder of the dusting powder base. If appropriate, volatile solvents can also

12

be employed as auxiliaries so that the active compounds are absorbed uniformly onto the dusting powder base.

#### G. Suspensions

Suspensions according to the invention contain the active compounds of the formula I and the corticosteroids in solid form, dispersed in a liquid phase. The particle size of the dispersed active compounds is within the limits of 0.1  $\mu\text{m}$  to about 100  $\mu\text{m}$ . Depending on the use, the solids content in a suspension according to the invention is between about 0.5% and 40%. Their fundamental property is said to be the slow sedimentation of the solid particles and the ease with which they can be shaken up, in order to avoid incorrect dosages.

Suspensions according to the invention are as a rule prepared by triturating the solid with a small amount of liquid and gradually diluting the mixture to the final volume. If the dispersing agent consists of several liquids (for example Mucilago Tylose, glycerol and water), the component with the highest viscosity is used for the trituration. The final mixture can be homogenized mechanically with the aid of mixers, homogenizers, corundum disc mills, bead mills, ultrasound and the like.

The following measures are taken to prepare stable suspensions according to the invention:

Increase in the wettability of the particles and controlled flocculation by addition of amphiphilic auxiliaries and suitable electrolytes.

Increase in the viscosity of the dispersing agent to reduce the rate of sedimentation.

As with emulsions, surfactants can be used to reduce the solid/liquid surface tension.

These are absorbed by the hydrophobic particles of the disperse phase so that their solvated hydrophobic molecular portions project away from the active compound into the dispersing agent. If ionic surfactants are used, the particles are also given a sufficient electrostatic charge, as well as being wetted well, which helps to stabilize the suspension.

Electrolytes such as phosphates, alkali metal carbonates, citrates, gallates and the like which determine the potential after adsorption onto the active compound particles lead to a high Zeta potential and thus have the effect of mutual repulsion.

Suspensions which can easily be shaken up again and can therefore be dosed uniformly can be prepared by means of the active compounds of the formula I and the corticosteroids by flocculating the originally hydrophobic particles initially negatively charged by an anionic surfactant by means of a calcium salt or aluminium salt. The loose, bulky sediment which forms can easily be redispersed by shaking.

The addition of macromolecular substances, such as methylcellulose, starch, tragacanth and the like has the effect of building up sorbate shells around the particles of the disperse phase, in addition to increasing the viscosity. This build-up of sorbate shells in turn impedes aggregation.

Gel-forming agents which impart thixotropic properties to the external phase are particularly effective. The gel structure formed in the resting state prevents sedimentation of the disperse phase. Under the action of weak shearing forces (shaking), the dispersion agent becomes free-flowing and the suspension can be easily dosed.

The statements made on aqueous suspensions also apply vice versa for oily suspensions. Surfactants, for

example, of the metallic soap type are indicated here, above all for processing oxophilic (hydrophilic) solids. However, the content of free fatty acids in the oil or a corresponding addition is frequently already sufficient for lyophilization of zinc oxide or other hydrophilic substances used in medicaments. The effect of an additive with such a wetting action can already be seen in the appearance, for example of a zinc oil: while zinc oxide which has not been wetted sediments cumulatively and gives a glossy supernatant of pure oil (above all when liquid paraffin is used), ZnO particles wetted with the aid of fatty acids form flocks, sediment little and impart a matt appearance to the surface.

#### H. Emulsions

The topical formulation, according to the invention, of the active compounds of the formula I and the corticosteroids can also be in the form of "liquid/liquid" disperse systems.

According to "Arzneiformenlehre" ("Drug form doctrine"), P. H. List, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart 1985, page 168, the following points should be taken into consideration for preparation of emulsions:

1. Type of emulsion required	oil-in-water, water-in-oil, bicoherent system
2. Viscosity	flowable or spreadable system
3. Temperature stability	stability in the widest possible temperature range, sterilizability
4. Degree of division	droplet size $> 1 \mu\text{m}$ for the usual emulsions
5. Constituents	choice of components for therapeutic, physiological, technological and economic reasons
6. Concentration ratios	for application technology reasons
7. Equipment and batch sizes	

Because of the large number of factors, it is not possible to draw up generally applicable preparation rules. Rather, series of experiments should be carried out, and for these the HLB system, the Lin process for preparation of oil-in-water emulsion, the plotting of three-component graphs or the formation of emulsifier gels may be helpful.

Emulsifiers and other auxiliaries for the preparation of topical formulations according to the invention are described in H. P. Fiedler, Lexikon der Hilfsstoffe für Pharmazie, Kosmetik und angrenzende Gebiete (Encyclopaedia of Auxiliaries for Pharmacy, Cosmetics and Related Fields), Editio Cantor K. G., Aulendorf i. Württ., 1971, in particular on pages 185-194.

Emulsions of the active compounds of the formula I in combination with the corticosteroids can be used externally. Both types of emulsion, that is to say oil-in-water and water-in-oil emulsions, can be used externally.

Liquid oil-in-water emulsion, which are often also called lotions, are used chiefly for dermatological purposes. The semi-solid forms are called washable ointments; as are the semi-solid water-in-oil emulsions called creams. Liquid water-in-oil emulsions for external use have also been given in DAB 6 under the name liniments.

#### I. Solutions

The topically applicable formulations, according to the invention, of the active compounds of the formula I and the corticosteroids include solutions.

The choice of a solvent or solvent mixture for the preparation of the formulations according to the invention depends primarily on the nature of the substance or substance mixture to be dissolved. Moreover, however, the question of whether all or some of the solvent remains in the finished formulation plays a role. In the case where it remains, the solvent must above all be physiologically acceptable.

For these reasons, only a relatively small number of liquids are suitable solvents for medicament preparation.

That used by far the most frequently is water.

Suitable solvents are therefore water and all water-miscible solvents. Possible solvents are, for example, alcohols, such as ethanol, isopropyl alcohol and propylene glycol, polyethylene glycols, glycerol, methylcellosolve, cellosolve, esters, morpholines, dioxane, dimethylsulphoxide and the like.

One or more solvents can be used to prepare the formulations according to the invention. Suitable solubilizing agents are, above all: surfactants, such as polyoxyethylated sorbitan fatty acid esters, polyoxyethylated fatty acid ethers and esters, and the like. Water-immiscible solvents are moreover also suitable, such as liquid esters and oils, in particular isopropyl myristate, isopropyl palmitate, 2-octyldodecanol, medium-chain triglycerides, adipic acid esters, sebacic acid esters, paraffin oil and silicone oil, and mixtures thereof, as well as water-miscible solvents and surfactants.

J. Ointments, pastes, creams, foams

(According to Ullmann, Volume 18, Pharmazeutische Technologie und Arzneiformenlehre (Pharmaceutical Technology and Drug Form Doctrine), P. H. List, 4th edition, Wissenschaftl. Verlagsgesellschaft mbH, Stuttgart).

Ointments are gels with a plastic deformability which can contain dissolved emulsified or suspended active compounds of the formula I and corticosteroids.

They are used for local treatment of diseased parts of the skin or mucous membrane. They can also fulfil the function of protecting and covering ointments here. If the ointments contain active compounds of the formula I and corticosteroids which pass into and penetrate the deeper-lying layers of skin and act there, they are called penetration ointments. Ointments in which the active compounds pass into the underskin tissue and finally into the blood circulation are called absorption ointments (percutaneous or transcutaneous absorption).

Ointments are called by different names, depending on their composition, their consistency or the site of application:

Unguent, ointments (French: pommades) is either the generic term for this medicament form or means anhydrous formulations based on various bases.

Cerates is the name for ointments which consist of a mixture of wax and oil.

Cremors and creams are ointments with a particularly soft consistency containing relatively large amounts of water.

Glycerols are glycerol-containing formulations with a semi-solid consistency.

Pastae and pastes are ointments with a high content of pulverulent solids and therefore a high consistency.



4,844,902

15

Oculents, ophthalmic unguents and eye ointments are soft ointments for application into the conjunctival sac and to the edges of the lids, on which particular requirements in respect of purity and particle size are imposed.

Ointments bases which are used for the topically applicable formulations of the invention are hydrocarbon gels, lipogels, hydrogels, polyethylene glycol gels and silicone gels.

Vaseline, Plastibase, waxes (according to the DGF), in particular beeswax, spermaceti DAB 8, cetaceum, woolwax DAB 8. Lanae Cera, oleyl oleate DAB 8. Oleyl oleas, isopropyl myristate, lard, hardened groundnut oil, glycerol, sorbitol solutions, low molecular weight polyethylene glycols, colloidal silicic acid, Aerosil, swellable clays, such as bentonite, potash soap, soft soap, Opodeldoc, starch, cellulose derivatives, polyacrylic acid, polyethylene glycol gels DAB 8 and silicone oils are preferably suitable.

The topical formulations according to the invention are used in ointment form as solution ointments, emulsion ointments and suspension ointments. They preferably contain antioxidants, such as avenol, avenex, conidendrin, norconidendrin, nordihydroguajaritic acid, tocopherol, ascorbic acid esters, for example the stearate, palmitate, myristate and laurate, 3-butyl-4-hydroxyanisole, hydroquinone, propyl gallate, citric acid, cis-methylmaleic acid, gallic acid esters, such as ethyl gallate and propyl gallate, Ionol, BHT and tetraoxydimethylbisphenyl (TDBP).

Pastes are highly concentrated suspensions with a flow limit for use on the skin or mucous membrane. They contain a large amount of insoluble powder dispersed in a liquid or ointment-like vehicle.

The pulverulent constituents with a particle size of not more than 100  $\mu\text{m}$  are gradually triturated with the dispersing agent, which if necessary is melted or softened on a waterbath, to give a uniform composition, which is homogenized to the highest possible degree with an ointment mill.

Creams in the sense of the invention are particularly smooth formulations which contain relatively large amounts of water in the form of oil-in-water or water-in-oil emulsions. The ratio of the aqueous to the oily phase determines the viscosity and the ease of spreading of a cream. Suitable bases, depending on the type of emulsion, are, for example, various grades of Lanette (sodium fatty alcohol sulphate), cetylstearyl alcohol or wool fat, woolwax alcohols and the like.

The topical formulations according to the invention can also be in the form of foams. Foams here are understood as being disperse "gaseous/liquid" systems (for the definitions, terms and preparation see "Arzneiformenlehre" ("Drug Forms Doctrine"), List).

The topical formulations according to the invention exhibit a broad antibacterial spectrum against Gram-positive and Gram-negative germs, in particular against Enterobacteriaceae, and anti-inflammatory properties; above all also against those germs which are resistant to various antibiotics, such as, for example, penicillins, cephalosporins, aminoglycosides, sulphonamides and tetracyclines, coupled with a low toxicity.

The formulations according to the invention are active against a very broad spectrum of microorganisms. With their aid, Gram-negative and Gram-positive bacteria and bacteria-like microorganisms can be combated and the diseases caused by these pathogens can be prevented, alleviated and/or cured.

16

The topical formulations according to the invention are particularly active against bacteria and bacteria-like microorganisms and sensitive processes as well as other corticosteroid indications. They are therefore particularly suitable in human and animal medicine for the prophylaxis and chemotherapy of local and, if appropriate, systemic infections caused by these pathogens.

For example, local and/or systemic diseases caused by the following pathogens or by mixtures of the following pathogens can be treated and/or prevented: Gram-positive cocci, for example Staphylococci (*Staph. aureus* and *Staph. epidermidis*) and Streptococci (*Strept. agalactiae*, *Strept. faecalis*, *Strept. pneumoniae* and *Strept. pyogenes*); Gram-negative cocci (*Neisseria gonorrhoeae*) and Gram-negative rod-shaped bacillae, such as Enterobacteriaceae, for example *Escherichia coli*, *Haemophilus influenzae*, *Citrobacter* (*Citrob. freundii* and *Citrob. diversus*), *Salmonella* and *Shigella*; and furthermore *Klebsiella* (*Klebs. pneumoniae* and *Klebs. oxytoca*), *Enterobacter* (*Ent. aerogenes* and *Ent. agglomerans*), *Hafnia*, *Serratia* (*Serratia marcescens*), *Proteus* (*Pr. mirabilis*, *Pr. rettgeri* and *Pr. vulgaris*), *Providencia* and *Yersinia*, and the genus *Acinetobacter*. The antibacterial spectrum moreover includes the genus *Pseudomonas* (*Ps. aeruginosa* and *Ps. maltophilia*) and strictly anaerobic bacteria, such as, for example, *Bacteroides fragilis*, representatives of the genus *Peptococcus*, *Peptostreptococcus* and the genus *Clostridium*; and furthermore *Mycoplasma* (*M. pneumoniae*, *M. hominis* and *M. urealyticum*) and *Mycobacteria*, for example *Mycobacterium tuberculosis*.

The above list of pathogens is merely by way of example and is in no way to be interpreted as limiting. Examples which may be mentioned of diseases which can be caused by the pathogens or mixed infections mentioned and can be prevented, alleviated or cured by the topically applicable formulations according to the invention are: infectious diseases in humans, such as, for example, septic infections, bone and joint infections, skin infections, postoperative wound infections, abscesses, phlegmons, wound infections, infected burns, burn wounds, infections in the oral region, infections following dental operations, septic arthritis, mastitis, tonsillitis, genital infections and eye infections.

As well as in humans, bacterial infections can also be treated in other species. Examples which may be mentioned are: pigs: *coli* diarrhoea, enterotoxaemia, sepsis, dysentery, salmonellosis, metritis-mastitis-agalactiae syndrome and mastitis; ruminants (cattle, sheep, goats): diarrhoea, sepsis, bronchopneumonia, salmonellosis, pasteurellosis, mykoplasmosis and genital infections; horses: bronchopneumonia, joint ill, puerperal and post-puerperal infections and salmonellosis; dogs and cats: bronchopneumonia, diarrhoea, dermatitis, otitis, urinary tract infections and prostatitis; and poultry (chickens, turkeys, quail, pigeons, ornamental birds and others): mycoplasmosis, *E. coli* infections, chronic respiratory tract diseases, salmonellosis, pasteurellosis, psittacosis.

Bacterial infections in the breeding and husbandry of stock and ornamental fish can also be treated, the antibacterial spectrum being extended beyond the pathogens mentioned above to other pathogens, such as, for example, *Pasteurella*, *Brucella*, *Campylobacter*, *Listeria*, *Erysipelothrix*, *Corynebacteria*, *Borellia*, *Trepone*, *Nocardia*, *Rickettsia* and *Yersinia*.

The present invention includes pharmaceutical formulations which, in addition to non-toxic, inert pharma-

4,844,902

17

ceutically suitable excipients, contain one or more compounds according to the invention or consist of one or more active compounds according to the invention, and to processes for the preparation of these formulations.

The present invention also includes pharmaceutical formulations in dosage units.

The abovementioned pharmaceutical formulations can also contain other pharmaceutically active compounds in addition to the compounds according to the invention.

The abovementioned pharmaceutical formulations are prepared in the customary manner by known methods, for example by mixing the active compound or compounds with the excipient or excipients.

### EXAMPLES

#### Adhesive topical formulations

##### 1. Example of a stick

#### Composition

1	Ciprofloxacin hydrochloride $\times 1 \text{ H}_2\text{O}$	0.2 g
2	Active compound X	1.0 g
3	Beeswax	12.0 g
4	Vaseline	6.0 g
5	Cetyl alcohol	4.0 g
6	Woolwax	3.0 g
7	Isopropyl myristate	9.0 g
8	Castor oil	64.8 g
		100.0 g

#### Preparation

Substances (3) to (8) are weighed and melted at about 70°, with stirring. Ciprofloxacin hydrochloride is suspended therein. The melt is poured into appropriate molds (sticks) and cooled to room temperature.

##### 2. Example of a protective ointment

#### Composition

1	Ciprofloxacin	0.5 g
2	Active compound H	0.5 g
3	Low molecular weight acrylic resin	5.0 g
4	Demineralized water	71.5 g
5	Ammonia	1.0 g
6	Woolwax alcohol	9.0 g
7	Tween 81 <sup>®</sup>	1.0 g
8	Liquid paraffin	11.5 g
		100.0 g

#### Preparation

(a) Heat substances 1, 2, 6, 7 and 8 to 65° C., with stirring.

(b) Heat substances 3 and 4 (aqueous phase) to 65° C.

(c) Incorporate b into a, with vigorous stirring.

(d) Add the ammonia to c, with vigorous stirring, and cool to room temperature, with stirring.

##### 3. Example of a film-forming concentrate

#### Composition

1	Ciprofloxacin	5.0 g
2	Active compound G <sup>1</sup>	1.0 g
3	Sodium alginate	9.0 g
4	Non-ionic emulsifier	2.0 g
5	Colloidal silicic acid	2.0 g
6	Isopropanol to 100 ml	81.0 g

18

-continued

100.0 g

#### Preparation

Ciprofloxacin and hydrocortisone acetate are dispersed in 3 and 5, with stirring. 2 and 4 are introduced into the dispersion and the mixture is homogenized.

Before use, this suspension is shaken up and diluted 1+9 with water. A gel forms which, after drying, forms an elastic film on the skin and sticks for a relatively long time, even in contact with water.

##### 4. Example of a solution

#### Composition

1	Ciprofloxacin or norfloxacin + 0.5 g of active compound H	0.03 g
2	Poly(methyl vinyl ether/monoalkyl maleate)	2.5 g
3	Isopropanol	96.97 g
		100.0 g

#### Preparation

The solution can be applied to the skin. After drying, a film containing the active compounds is formed on the skin.

##### 5. Example of a spray formulation

The solution from Example 4 is introduced into suitable aerosol cans with propane/butane as the propellant gas in a ratio of 1+2.

#### Plasters

##### Example 1

A 12.5% strength polyisobutylene solution (viscosity-average molecular weight 1,270,000) (in benzene) is applied to siliconized paper, a knitted fabric consisting of polyamide-polyurethane fibers is laminated on and the system is dried in a drying tunnel in zones at 70° / 90° / 100° C. (polymer 30 g/m<sup>2</sup>).

A mixture consisting of 36,000 g of polyisobutylene of viscosity-average molecular weight 400,000, 44,928 g of light liquid paraffin, 9,000 g of polyterpene resin from  $\beta$ -pinene, 10,000 g of ciprofloxacin or ofloxacin or norfloxacin and 0.072 g of an antiageing agent, dissolved in benzene/acetone, is applied to siliconized paper and the paper is dried in a drying tunnel in zones at 70° / 90° / 100° C. (active compound release system about 150 g/m<sup>2</sup>).

After drying, the polyisobutylene-impregnated knitted fabric with a stretch character is laminated on.

##### Example 2

A polymer solution (benzene/acetone) consisting of 36,000 g of polyisobutylene of viscosity-average molecular weight 1,270,000, 44,928 g of light liquid paraffin, 9,000 g of polyterpene resin from  $\alpha$ -pinene, 5,000 g of ciprofloxacin and 5,000 g of active compound N and 0.072 g of anti-ageing agent was applied to siliconized paper and the paper was dried in a drying tunnel in zones at 70° / 90° / 100° C.

After drying, the active compound release system was laminated with polyisobutylene (as in Example 1) on coated stretch material.



4,844,902

## 19

The plasters according to the invention have just as good an absorption of the active compounds as conventional plasters coated with aluminum-polyethylene films.

The methods and materials described in Koch-Satlow, Grosses Textillexikon (Large Textile Encyclopaedia) are used, inter alia, for laminating the active compound depot onto the textile sheet-like structure with a stretch character.

## Gels

## Example 1

Ciprofloxacin lactate	0.20 g
Active compound prednisolone	5.00 g
Benzyl alcohol	3.00 g
Hydroxypropylcellulose	2.50 g
(molecular weight 1,000,000)	
Demineralized water to	100 g

## Example 2

Ciprofloxacin acetate	0.20 g
Hydrocortison acetate	0.25 g
Benzyl alcohol	3.00 g
Hydroxypropylcellulose	2.5 g
(molecular weight 1,000,000)	
Demineralized water to	100 g

## Example 3

Norfloxacin hydrochloride	0.20 g
Prednisolone	5.00 g
Benzyl alcohol	3.00 g
Hydroxypropylcellulose	2.5 g
(molecular weight 1,000,000)	
Demineralized water to	100 g

## Example 4

Ofloxacin hydrochloride	0.20 g
Prednisolone	5.00 g
Benzyl alcohol	3.00 g
Hydroxypropylcellulose	17.50 g
(molecular weight 60,000)	
Demineralized water to	100 g

## Creams

## Example 1

## oil-in-water cream

## Phase I

Sorbitan monostearate	2.0 g
Polyoxyethylene (20) sorbitan monostearate	1.5 g
Synthetic spermaceti	3.0 g
Cetyl stearyl alcohol	10.0 g
2-Octyldodecanol	13.5 g

Heat to 75° C., stir and mix.

## Phase II

Ciprofloxacin, norfloxacin or	1.0 g
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## 20

## -continued

ofloxacin, enoxacin, pefloxacin

in combination with 1 to 2 g of active compounds A to Z and A<sup>1</sup> to H<sup>1</sup>. Add to phase I, stir and suspend. Phase III

Benzyl alcohol	1.0 g
Demineralized water	68.0 g

Heat to 75° C. and add to phase II. Mix intensively and cool slowly to room temperature, with further stirring. Homogenize.

## Example 2

## oil-in-water cream

## Phase I

Sorbitan monostearate	1-3 g
Polyoxyethylene (20) sorbitan monostearate	0.5-2.5 g
Synthetic spermaceti	2-4 g
Cetyl stearyl alcohol	5-15 g
Isopropyl myristate	5-25 g

Heat to 75° C., stir and mix.

## Phase II

Add ciprofloxacin, norfloxacin or ofloxacin	0.5-1.5 g
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and active active compounds A to Z or A<sup>1</sup> to H<sup>1</sup> in just such amounts to phase I, stir and suspend.

## Phase III

Benzyl alcohol	0.5-1.5 g
Demineralized water	quant. sat.

Heat to 75° C. and add to phase II. Mix intensively and cool slowly to room temperature, with further stirring. Homogenize.

## Liquid plasters

## Example 1

Flupamesone	1.0 g
Ciprofloxacin	1.0 g
Benzyl alcohol	5.0 g
Hydroxypropylcellulose	10.0 g
(molecular weight 60,000)	
Isopropanol to	100 ml

## Example 2

Triamcinolone	0.1 g
Ciprofloxacin	0.1 g
Benzyl alcohol	5.0 g
Isopropyl myristate	6.0 g
Hydroxypropylcellulose	10.0 g
(molecular weight 60,000)	
Isopropanol to	100 ml

## 21

4,844,902

## Example 3

Fluocinolone acetonide	1.0 g
Ciprofloxacin	1.0 g
Benzyl alcohol	4.0 g
Isopropyl stearate	10.0 g
Hydroxypropylcellulose (molecular weight 60,000)	12.0 g
Isopropanol to	100 ml

## Example 4

Betamethasone valerate	1.0 g
Ciprofloxacin	1.0 g
1,2-Propylene glycol	1.0 g
Isopropyl myristate	6.0 g
Hydroxypropylcellulose (molecular weight 60,000)	10.0 g
Isopropanol to	100 ml

## Example 5

Norfloxacin	0.1 g
Benzyl alcohol	5.0 g
Isopropyl myristate	6.0 g
Hydroxypropylcellulose (molecular weight 60,000)	10.0 g
Isopropanol	to 100 ml

## Example 6

Ofloxacin	1.0 g
Benzyl alcohol	5.0 g
Isopropyl myristate	6.0 g
Hydroxypropylcellulose (molecular weight 60,000)	10.0 g
Isopropanol	to 100 ml

## Example 7

Prednisolone	1.0 g
Ciprofloxacin	1.0 g
Benzyl alcohol	8.0 g
Isopropyl myristate/isopropyl stearate/ isopropyl palmitate	1.0 g
Hydroxypropylcellulose (molecular weight 60,000)	10.0 g
Isopropanol	to 100 ml

## Example 8

Prednisolone	1.0 g
Norfloxacin	1.0 g
Benzyl alcohol	5.0 g
Isopropyl myristate	6.0 g
Methylcellulose	10.0 g
Isopropanol	to 100 ml

## Sprays

The active compound solutions or suspensions prepared according to Example 1 to 8 can also be processed to sprays. For this purpose, for example, a 60 to 90% active compound solution is mixed with 20 to 40%

## 22

of the usual propellants, for example N<sub>2</sub>, N<sub>2</sub>O, CO<sub>2</sub>, propane, butane, halogenohydrocarbons and the like.

## Emulsions/Creams

## Example 1

## oil-in-water emulsion

## Phase I

"Glyceryl stearates" (mixture of mono- and diglycerides of palmitic and stearic acid)	8.00 g
2-Octyldodecanol	10.00 g
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	1.50 g
Cetyl stearyl alcohol with about 30 mols of ethylene oxide	1.50 g
Heavy liquid paraffin	6.00 g
1,2-Propylene glycol	5.00 g
Caprylic/capric acid triglyceride	6.00 g

The mixture is stirred and melted at 70° C.

## Phase II

Demineralized water	58.00 g
Phase III	1.00 g
Prednisolone	1.00 g
Ciprofloxacin	3.00 g
suspended in benzyl alcohol	

Phase II is heated to 75° C. and phase I, which has been heated to 70° C., is added, with stirring. The mixture is allowed to cool slowly to 40° C., phase III is added and the mixture is allowed to cool to room temperature, with stirring. The crude emulsion is homogenized at 20° to 25° C. in a high pressure homogenizer. Examples 2 and 3 are processed in an analogous manner.

## Example 2

## oil-in-water emulsion

Desoxycorticosterone	5.00 g
Ciprofloxacin	1.00 g
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	8.00 g
Diglycerides of palmitic and stearic acid	9.00 g
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	3.00 g
2-Octyldodecanol	10.00 g
Heavy liquid paraffin	5.00 g
Benzyl alcohol	5.00 g
Demineralized water	to 100 ml

## Example 3

## oil-in-water emulsion

Desoxycorticosterone	5.00 g
Norfloxacin	1.00 g
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	9.00 g
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	3.00 g
2-Octyldodecanol	10.00 g
Benzyl alcohol	5.00 g
Isopropyl myristate	5.00 g
Demineralized water	to 100 ml

4,844,902

23

## Example 4

oil-in-water cream, soft consistency

Prednisolone, fluocinolone acetonide, hydrocortisone acetate	1.00 g	5
Ciprofloxacin, norfloxacin, ofloxacin	1.00 g	
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	4.00 g	
Cetyl palmitate	4.00 g	10
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	1.00 g	
Cetyl stearyl alcohol with about 30 mols of ethylene oxide	1.00 g	
Isopropyl myristate/isopropyl palmitate, isopropyl stearate mixture	5.00 g	
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.50 g	15
45% strength sodium hydroxide	0.11 g	
Glycerol	3.00 g	
Benzyl alcohol	3.00 g	
Demineralized water	to 100 ml	20

## Example 5

oil-in-water cream, soft consistency

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup> in each case	1.00 g	25
Ciprofloxacin, norfloxacin, ofloxacin	1.00 g	
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	4.00 g	30
Cetyl palmitate	4.00 g	
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	1.00 g	
Cetyl stearyl alcohol with about 30 mols of ethylene oxide	1.00 g	
Isopropyl myristate/isopropyl palmitate/isopropyl stearate mixture	5.00 g	35
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.50 g	
45% strength sodium hydroxide	0.11 g	
Glycerol	3.00 g	
Benzyl alcohol	3.00 g	40
Demineralized water	to 100 ml	

## Example 6

oil-in-water cream, soft consistency

Hydrocortisone acetate	1.00 g	45
Norfloxacin, ofloxacin	1.00 g	
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	4.00 g	50
Stearic acid	4.00 g	
Cetyl palmitate	4.00 g	
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	1.00 g	
Cetyl stearyl alcohol with about 30 mols of ethylene oxide	1.00 g	55
Isopropyl myristate/isopropyl palmitate/isopropyl stearate mixture	5.00 g	
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.50 g	
45% strength sodium hydroxide	0.11 g	
Glycerol	3.00 g	
Benzyl alcohol	3.00 g	
Demineralized water	to 100 ml	

## Example 7

oil-in-water emulsion, non-greasy

Phase I

24

Decyl oleate	2.50 g
Isopropyl myristate	2.50 g
Light liquid paraffin	4.00 g
Polyethylene stearate	0.90 g
Fatty acid esters of sorbitan and glycerol	0.60 g

The mixture is stirred and melted at 70° C. for 10 minutes.

## Phase II

Demineralized water	50.00 g
Allantoin	0.10 g

## Carbopol mucilage

Denatured alcohol	10.00 g
Carbopol 934 (weakly crosslinked polyacrylic acid)	0.70 g
Demineralized water	22.945 g

The components are dispersed with a Turrax and the dispersion is left to swell for 2 hours and then neutralized with 0.155 g of 45% strength sodium hydroxide solution.

Phase II is heated to 75° C. and phase I, which has been heated to 70° C., is added, with stirring, and the mixture is cooled to 45° C.. Stir in the Carbopol mucilage at 45° C. and cool further to 40° C.. Add 1.00 g of collagen at 40° C. and cool to 25° C.. Incorporate 1.0 g of ciprofloxacin into 3.0 g of benzyl alcohol and add to phase I and II.

The crude emulsion is then homogenized at 20° C. to 25° C. in a high pressure homogenizer, with stirring.

Examples 8, 9, 10 and 11 are processed in an analogous manner.

## Example 8

oil-in-water emulsion, non-greasy

Hydrocortisone acetate	0.20 g	45
Ciprofloxacin	0.10 g	
Decyl oleate	2.50 g	
Isopropyl myristate	2.50 g	
Light liquid paraffin	4.00 g	
Polyethylene stearate	0.90 g	
Fatty acid esters of sorbitan and glycerol	0.60 g	
Allantoin	0.155 g	50
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.70 g	
Collagen	1.00 g	
Benzyl alcohol	3.00 g	
Ethanol	10.00 g	
Demineralized water	to 100 ml	55

## Example 9

oil-in-water emulsion, non-greasy

Hydrocortisone acetate	0.15 g	60
Norfloxacin	0.15 g	
Decyl oleate	2.50 g	
Isopropyl myristate	2.50 g	
Light liquid paraffin	4.00 g	
Polyethylene stearate	0.90 g	
Fatty acid esters of sorbitan and glycerol	0.60 g	65
Allantoin	0.10 g	
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.70 g	

4,844,902

25

-continued

45% strength sodium hydroxide	0.155 g	
Collagen	1.00 g	
Benzyl alcohol	3.00 g	
Ethanol	10.00 g	5
Demineralized water to	100 ml	

Example 10

oil-in-water emulsion, non-greasy

Hydrocortisone acetate	0.10 g	
Ofloxacin	0.10 g	
Decyl oleate	2.50 g	
Isopropyl myristate	2.50 g	15
Light liquid paraffin	4.00 g	
Polyethylene stearate	0.90 g	
Fatty acid esters of sorbitan and glycerol	0.60 g	
Allantoin	0.10 g	
45% strength sodium hydroxide	0.155 g	20
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.70 g	
Collagen	1.00 g	
Benzyl alcohol	3.00 g	
Ethanol	10.00 g	
Perfume oil	0.60 g	
Demineralized water to	100 ml	

Example 11

oil-in-water emulsion, non-greasy

Hydrocortisone acetate, prednisolone in each case	0.05 g	
Ciprofloxacin	0.05 g	
Decyl oleate	2.50 g	
Isopropyl myristate	2.50 g	35
Light liquid paraffin	4.00 g	
Polyethylene stearate	0.90 g	
Fatty acid esters of sorbitan and glycerol	0.60 g	
Allantoin	0.10 g	
45% strength sodium hydroxide	0.155 g	40
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.70 g	
Collagen	1.00 g	
Benzyl alcohol	3.00 g	
Ethanol	10.00 g	
Demineralized water to	100 ml	

Example 12

oil-in-water emulsion, non-greasy

Triamcinolone	0.50 g	
Ciprofloxacin	0.10 g	
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	4.00 g	
Cetyl palmitate	4.00 g	
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	1.00 g	
Cetyl stearyl alcohol with about 30 mols of ethylene oxide	1.00 g	
Isopropyl myristate/isopropyl palmitate/isopropyl stearate mixture	5.00 g	55
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.50 g	
45% strength sodium hydroxide	0.11 g	
Glycerol	3.00 g	60
Benzyl alcohol	3.00 g	
Demineralized water to	100 ml	

26

Example 13

oil-in-water cream, soft consistency

Flupamesone	0.05 g	
Ofloxacin	0.05 g	
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	4.00 g	
Cetyl palmitate	4.00 g	
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	1.00 g	10
Cetyl stearyl alcohol with about 30 mols of ethylene oxide	1.00 g	
Isopropyl myristate/isopropyl palmitate/isopropyl stearate mixture	5.00 g	
Weakly crosslinked polyacrylic acid of extremely high molecular weight	0.50 g	15
45% strength sodium hydroxide	0.11 g	
Glycerol	3.00 g	
Benzyl alcohol	3.00 g	
Demineralized water to	100 ml	

Example 14

oil-in-water cream, soft consistency

Prednisolone	5.00 g	
Norfloxacin, pefloxacin	1.00 g	
"Glyceryl stearates" mixture of mono- and diglycerides of palmitic and stearic acid	4.00 g	
Na stearate	16.00 g	
Cetyl stearyl alcohol with about 12 mols of ethylene oxide	3.00 g	30
Benzyl alcohol	56	
2-Octyldodecanol	3.50 g	
Coconut fatty acid isopropyl ester	2.50 g	
(Isopropyl myristate/isopropyl palmitate, isopropyl stearate mixture)	2.50 g	
Light liquid paraffin	3.00 g	35
Demineralized water to	100 ml	

Gels

Example 1

Phase I  
Dissolve

Hydrocortisone acetate	1.00 g	
Ofloxacin	1.00 g	
in isopropanol	40.00 g	
subsequently stir in		
Polyol fatty acid ester	4.00 g	
Benzyl alcohol	3.00 g	50
Diisopropyl adipate	4.00 g	
and dissolve.		

Phase II  
Introduce into

demineralized water	46.10 g	
Carbopol 940	1.50 g	
with stirring, allow to swell for about 2 hours and neutralize with		
45% strength NaOH	0.40 g	60

Incorporate phase I slowly in portions into phase II, with stirring.

Example 2

Flupamesone	1.00 g
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4,844,902

27

-continued

Ciprofloxacin	1.00 g
Polyol fatty acid ester	4.00 g
Isopropyl myristate	4.00 g
Benzyl alcohol	1.00 g
Isopropanol	45.00 g
Carbopol 940	1.50 g
45% strength NaOH	0.40 g
Demineralized water	43.10 g

## Example 3

Fluocinolone acetonide	1.00 g
Ofloxacin	1.00 g
Polyol fatty acid ester	4.00 g
Coconut fatty acid isopropyl ester	4.00 g
Benzyl alcohol	5.00 g
Isopropanol	45.00 g
Carbopol 940	1.50 g
45% strength NaOH	0.40 g
Demineralized water	39.10 g

## Greasy ointment

## Example 1

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	1.00 g
Ciprofloxacin	1.00 g
Woolwax alcohol	5.00 g
White vaseline to	100 g

## Dusting powder

## Example 1

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	0.1 g
Ciprofloxacin hydrochloride	0.1 g
Lactose to	100 g

## Shaking mixture

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	1.0 g
Ciprofloxacin	1.0 g
Lanette N	3.0 g
Zinc oxide	18.0 g
Talc	18.0 g
85% strength glycerol	18.0 g
96% strength ethanol	13.2 g
Demineralized water	28.8 g

The ciprofloxacin, zinc oxide and talc are suspended in a solution of the other auxiliaries.

## Paste

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	10.0 g
Ciprofloxacin	10.0 g
Wheat starch	20.0 g
Zinc oxide	20.0 g
White vaseline	50.0 g

The solids are dried at 40° C. for 4 hours, sieved and suspended in the molten vaseline and the suspension is stirred until cold.

28

## Polyethylene glycol ointment

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	0.50 g
Ciprofloxacin	0.50 g
Polyethylene glycol 300	49.75 g
Polyethylene glycol 1500	49.75 g

The ciprofloxacin is suspended in the melt of the polyethylene-glycols. The suspension is then stirred until cold.

## Greasy ointment

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	1.00 g
Microfine ciprofloxacin	1.00 g
Methyl p-hydroxybenzoate	0.07 g
Propyl p-hydroxybenzoate	0.03 g
Heavy liquid paraffin	15.00 g
Woolwax alcohol ointment	83.90 g

The ciprofloxacin is introduced into the molten greasy phase. The mixture is then cooled, with stirring.

## Dusting powder

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	0.01 g
Ciprofloxacin, sodium salt	0.01 g
Non-swellable rice starch	99.99 g

## Water-in-oil ointment

Active compounds A to Z or A <sup>1</sup> to H <sup>1</sup>	2.00 g
Ciprofloxacin	2.00 g
Protegin X	22.00 g
Beeswax	3.00 g
Isopropyl myristate	1.50 g
Medium-chain triglycerides	1.50 g
Mixture of alkyl-branched fatty acid esters	2.00 g
Glycerol	3.00 g
Demineralized water	65.00 g

Other examples of formulations with a particular degree of penetration.

## Example 1

Triamcinolone acetonide	1.0%
(triamcinolone below)	
Ciprofloxacin hydrochloride	1.0%
Propylene glycol (1,2-propanediol)	94.0%
Methyl laurate	4.0%

## Example 2

Hydrocortisone acetate	1.0%
Ciprofloxacin lactate	1.0%
Propylene glycol (1,2-propanediol)	93.0%
Oleic acid	5.0%

## Example 3

Betamethasone valerate	0.5%
Ciprofloxacin	0.5%
Propylene glycol (1,2-propanediol)	93.0%

4,844,902

29

-continued

Oleyl alcohol	6.0%
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## Example 4

Fluocinolone acetonide	0.5%
Ciprofloxacin	0.5%
Propylene glycol (1,2-propanediol)	94.0%
Monoolein	5.0%

## Example 5

Flupamesone	0.5%
Ciprofloxacin	0.5%
Propylene glycol (1,2-propanediol)	97.0%
Myristyl alcohol	2.0%

## Example 6

Triamcinolone	0.5%
Ofloxacin	0.5%
1,2-Butanediol	95.0%
Methyl laurate	4.0%

## Example 7

Triamcinolone	0.5%
Norfloxacin	0.5%
1,3-Butanediol	97.0%
Methyl laurate	2.0%

## Example 8

Hydrocortisone acetate	0.25%
Ciprofloxacin	0.25%
1,2-Butanediol	97.50%
Oleic acid	2.0%

## Example 9

Hydrocortisone acetate	2.0%
Norfloxacin	2.0%
1,3-Butanediol	91.0%
Oleic acid	5.0%

## Example 10

Betamethasone valerate	2.0%
Pefloxacin, ofloxacin	2.0%
1,2-Butanediol	91.0%
Oleyl alcohol	5.0%

## Example 11

Fluocinolone acetonide	5.0%
Ciprofloxacin	5.0%
1,2-Butanediol	87.0%
Monoolein	3.0%

30

## Example 12

Flupamesone	1.0%
Ciprofloxacin	1.0%
Hydrocortisone acetate	1.0%
Propylene glycol (1,2-propanediol)	92.0%
Myristyl alcohol	5.0%

## Example 13

Desoxycorticosterone	5.0%
Ciprofloxacin, ofloxacin	5.0%
Propylene glycol (1,2-propanediol)	85.0%
Oleic acid	5.0%

## Example 14

Prednisolone	5.0%
Ciprofloxacin	5.0%
Propylene glycol (1,2-propanediol)	86.0%
Myristyl alcohol	4.0%

## Example 15

Prednisone	2.0%
Ciprofloxacin	2.0%
1,2-Butanediol	52.0%
Oleic acid	4.0%
Ethanol	40.0%

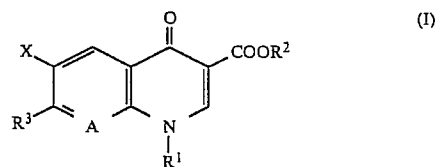
## Example 16

Methylprednisolone	4.0%
Ciprofloxacin	4.0%
1,3-Butanediol	51.0%
Oleyl alcohol	1.0%
Isopropanol	40.0%

It will be appreciated that the instant specification and examples are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

I claim:

1. A topically applicable formulation comprising by weight about 0.05 to 30% of an antibacterially active compound of the formula



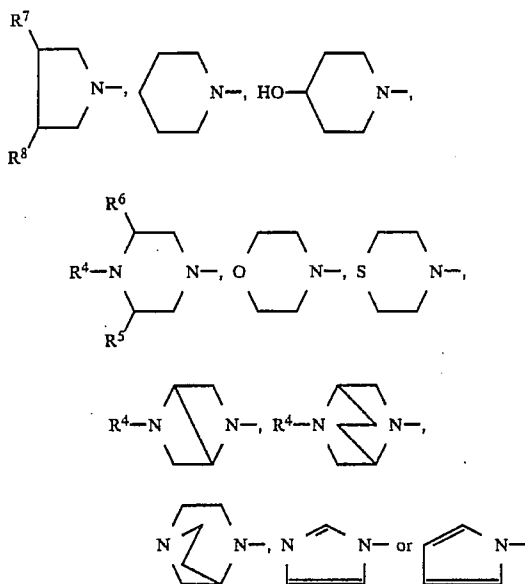
in which

R<sup>1</sup> represents methyl, ethyl, propyl, isopropyl, cyclopropyl, vinyl, 2-hydroxyethyl, 2-fluoroethyl, methoxy, amino, methylamino, dimethylamino, ethylamino, phenyl, 4-fluorophenyl or 2,4-difluorophenyl,

4,844,902

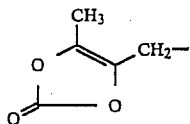
31

R<sup>2</sup> represents hydrogen, alkyl with 1 to 4 carbon atoms or (5-methyl-2-oxo-1,3-dioxol-4-yl)-methyl, R<sup>3</sup> represents methyl or a cyclic amino group of the formula



wherein

R<sup>4</sup> represents hydrogen, alkyl with 1 to 4 carbon atoms, 2-hydroxyethyl, allyl, propargyl, 2-oxopropyl, 3-oxobutyl, phenacyl, formyl, CFCL<sub>2</sub>-S-, CFCL<sub>2</sub>-SO<sub>2</sub>-, CH<sub>3</sub>O-CO-S-, benzyl, 4-aminobenzyl or



R<sup>5</sup> represents hydrogen or methyl,

R<sup>6</sup> represents hydrogen, alkyl with 1 to 4 carbon atoms, phenyl or benzyloxymethyl,

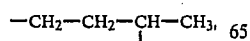
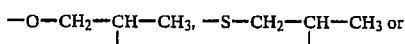
R<sup>7</sup> represents hydrogen, amino, methylamino, ethylamino, aminomethyl, methylaminomethyl, ethylaminomethyl, dimethylaminomethyl, hydroxyl or hydroxymethyl and

R<sup>8</sup> represents hydrogen, methyl, ethyl or chlorine, X represents hydrogen, fluorine, chlorine or nitro and A represents N or C-R<sup>9</sup>,

wherein

R<sup>9</sup> represents hydrogen, halogen, such as fluorine or chlorine, methyl or nitro, or

A, together with R<sup>1</sup>, can also form a bridge with the structure



0.01 to 10% of a corticosteroid, and a carrier.

32

2. A topically applicable formulation according to claim 1, wherein the antibacterially active compound is selected from the group consisting of ciprofloxacin, norfloxacin, perfloxacin, amifloxacin, pifloxacin, ofloxacin and enoxacin, and the corticosteroid is selected from the group consisting of

- A beclomethasone dipropionate
- B clobetasol propionate
- C diflucortolone valerate
- D fluocinolone acetonide
- E beclomethasone dipropionate
- F betamethasone benzoate
- G betamethasone dipropionate
- H betamethasone valerate
- I desonide
- J desoxymethasone
- K diflorasone diacetate
- L diflucortolone valerate
- M flucilorolone acetonide
- N fluocinolone acetonide
- O fluocinonide
- P fluocortolone
- Q fluprednidene (fluprednylidene) acetate
- R flurandrenolone
- S halcinonide
- T hydrocortisone butyrate
- X triamcinolone acetonide
- Y clobetasone butyrate
- Z flumethasone pivalate
- A<sup>1</sup> fluocinolone acetonide
- B<sup>1</sup> fluocortine butyl ester
- C<sup>1</sup> fluocortolone
- D<sup>1</sup> flurandrenolone
- E<sup>1</sup> hydrocortisone (urea)
- F<sup>1</sup> dexamethasone
- G<sup>1</sup> hydrocortisone (alcohol or acetate) and
- H<sup>1</sup> methylprednisolone.

3. A topically applicable formulation according to claim 1, in the form of a solution, spray, lotion, gel, ointment, cream, powder, dusting powder spray, paste, suspension, emulsion, foam.

4. A topically applicable formulation according to claim 1, comprising by weight about

(a<sup>1</sup>) 0.1 to 20% of an active compound of the formula

I,  
(a<sup>2</sup>) 0.01 to 10% of a corticosteroid,  
(b) 1 to 40% of a water-soluble gel- or lacquer-forming polymer,

(c) 40 to 98% of an organic water-miscible solvent which evaporates faster than water and in which the polymer does not dissolve, and

(d) 0.1 to 10% of at least one of a plasticizer, suspending auxiliary, antioxidant, spreading agent or dye-stuff.

5. A topically applicable formulation according to claim 1, comprising by weight about

(a<sup>1</sup>) 0.1 to 5% of an active compound of the formula

I,  
(a<sup>2</sup>) 0.02 to 5% of a corticosteroid,  
(b) 1 to 20% of a water-soluble gel- or lacquer-forming polymer,

(c) 60 to 90% of an organic water-miscible solvent which evaporates faster than water and in which the polymer does not dissolve, and

(d) 0.1 to 10% of at least one of a plasticizer, suspending auxiliary, antioxidant, spreading agent or dye-stuff.

4,844,902

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6. A topically applicable formulation according to claim 1, further containing a spreading agent.

7. A composition according to claim 1 in the form of a gel, the carrier including as a gel-forming agent, cellulose ether, polyacrylic acid, polymethacrylic acid, sodium alginate or propylene glycol alginate, sodium amylopectin semiglycolate, alginic acid, gum arabic or guar gum.

8. A composition according to claim 7 further containing a linear high molecular weight polysaccharide as a stabilizing gel-forming agent.

34

9. A composition according to claim 1 in the form of a suspension having solid particles of 0.1 to 100  $\mu\text{m}$  and a solids content of about 0.5 to 40% by weight.

10. A composition according to claim 1 in the form of an emulsion.

11. A composition according to claim 1 in the form of a solution, the carrier comprising at least one solvent selected from the group consisting of ethanol, isopropyl alcohol, propylene glycol, polyethylene glycol, glycerol, methylcellosolve, cellosolve, an ester, morpholine, dioxane, dimethylsulphoxide, water and cyclohexanone.

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UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 4,844,902

Page 1 of 2

DATED : Jul. 4, 1989

INVENTOR(S) : Grohe

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page, under "U.S. Patent Documents", line 1	Correct spelling of --Grohe--
Title Page, under " U.S. Patent Documents", line 2	Correct spelling of --Marples--
Col. 2, line 44	Delete "topicallys" and substitute --corticosteroids--
Col. 4, line 47	Delete "the" in first instance and substitute --to--
Col. 5, line 41	Delete "alcoholk" and substitute --alcohol;--
Col. 6, line 57	Delete "C <sub>18</sub> " in first instance and substitute --C <sub>12</sub> --
Col. 8, line 35	Correct spelling of --hydrogenated--
Col. 9, lines 18 and 19	Delete "to woven fabrics, knitted fabrics and"
Col. 10, line 51	Correct spelling of --corticosteroid--
Col. 12, line 64	Delete "dispersion" and substitute --dispersing--
Col. 16, line 6	Correct spelling of --local--
Col. 16, line 15	Correct spelling of --gonorrhoeae--
Col. 18, line 65	Delete "100" and substitute --110--
Col. 22, line 26	Delete "1.00g"
Col. 22, line 28	Delete "3.00 g" and substitute --1.00 g--
Col. 22, line 29	Insert --3.00 g--

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 4,844,902

Page 2 of 2

DATED : Jul. 4, 1989

INVENTOR(S) : Grohe

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 23, line 49

Correct spelling of --hydrocort-  
isone--

Col. 24, line 47

Correct spelling of --light--

Signed and Sealed this  
Twenty-seventh Day of November, 1990

*Attest:*

HARRY F. MANBECK, JR.

*Attesting Officer*

*Commissioner of Patents and Trademarks*

EXHIBIT E  
CIPRODEX<sup>®</sup> LABEL

Your Search Terms: PATENT BAYER

Version 1 - Published Mar 13, 2008

### **CIPRODEX - ciprofloxacin hydrochloride and dexamethasone suspension**

Alcon, Inc.

#### **Ciprodex®**

**(ciprofloxacin 0.3% and**

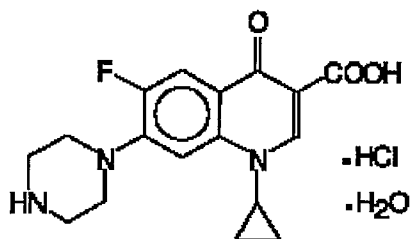
**dexamethasone 0.1%)**

**Sterile Otic Suspension**

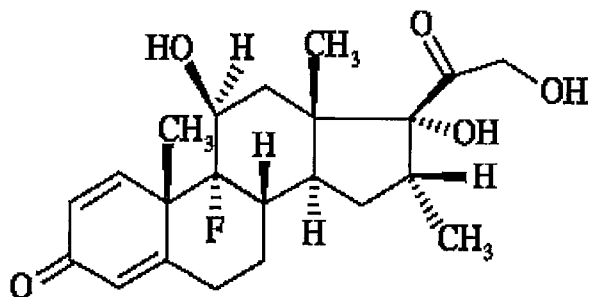
#### **DESCRIPTION**

CIPRODEX® (ciprofloxacin 0.3% and dexamethasone 0.1%) Sterile Otic Suspension contains the synthetic broad-spectrum antibacterial agent, ciprofloxacin hydrochloride, combined with the anti-inflammatory corticosteroid, dexamethasone, in a sterile, preserved suspension for otic use. Each mL of CIPRODEX® Otic contains ciprofloxacin hydrochloride (equivalent to 3 mg ciprofloxacin base), 1 mg dexamethasone, and 0.1 mg benzalkonium chloride as a preservative. The inactive ingredients are boric acid, sodium chloride, hydroxyethyl cellulose, tyloxapol, acetic acid, sodium acetate, edetate disodium, and purified water. Sodium hydroxide or hydrochloric acid may be added for adjustment of pH.

Ciprofloxacin, a fluoroquinolone is available as the monohydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. The empirical formula is  $C_{17}H_{18}FN_3O_3 \cdot HCl \cdot H_2O$  and the structural formula is:



Dexamethasone, 9-fluoro-11(beta),17,21-trihydroxy-16(alpha)-methylpregna-1,4-diene-3,20-dione, is an anti-inflammatory corticosteroid. The empirical formula is  $C_{22}H_{29}FO_5$  and the structural formula is:



#### **CLINICAL PHARMACOLOGY**

##### **Pharmacokinetics**

Following a single bilateral 4-drop (total dose = 0.28 mL, 0.84 mg ciprofloxacin, 0.28 mg dexamethasone) topical otic dose of CIPRODEX® Otic to pediatric patients after tympanostomy tube insertion, measurable plasma concentrations of ciprofloxacin and dexamethasone were observed at 6 hours following administration in 2 of 9 patients and 5 of 9 patients, respectively.

Mean  $\pm$  SD peak plasma concentrations of ciprofloxacin were  $1.39 \pm 0.880$  ng/mL (n=9). Peak plasma concentrations ranged from 0.543 ng/mL to 3.45 ng/mL and were on average approximately 0.1% of peak plasma concentrations achieved with an oral dose of 250-mg [1]. Peak plasma concentrations of ciprofloxacin were observed within 15 minutes to 2 hours post dose application.

Mean  $\pm$  SD peak plasma concentrations of dexamethasone were  $1.14 \pm 1.54$  ng/mL (n=9). Peak plasma concentrations ranged from 0.135 ng/mL to 5.10 ng/mL and were on average approximately 14% of peak concentrations reported in the literature following an oral 0.5-mg tablet dose [2]. Peak plasma concentrations of dexamethasone were observed within 15 minutes to 2 hours post dose application.

Dexamethasone has been added to aid in the resolution of the inflammatory response accompanying bacterial infection (such as otorrhea in pediatric patients with AOM with tympanostomy tubes).

### Microbiology

Ciprofloxacin has *in vitro* activity against a wide range of gram-positive and gram-negative microorganisms. The bactericidal action of ciprofloxacin results from interference with the enzyme, DNA gyrase, which is needed for the synthesis of bacterial DNA. Cross-resistance has been observed between ciprofloxacin and other fluoroquinolones. There is generally no cross-resistance between ciprofloxacin and other classes of antibacterial agents such as beta-lactams or aminoglycosides.

Ciprofloxacin has been shown to be active against most isolates of the following microorganisms, both *in vitro* and clinically in otic infections as described in the **INDICATIONS AND USAGE** section.

#### **Aerobic and facultative gram-positive microorganisms**

*Staphylococcus aureus*  
*Streptococcus pneumoniae*

#### **Aerobic and facultative gram-negative microorganisms**

*Haemophilus influenzae*  
*Moraxella catarrhalis*  
*Pseudomonas aeruginosa*

### INDICATIONS AND USAGE

CIPRODEX® Otic is indicated for the treatment of infections caused by susceptible isolates of the designated microorganisms in the specific conditions listed below:

**Acute Otitis Media** in pediatric patients (age 6 months and older) with tympanostomy tubes due to *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa*.

**Acute Otitis Externa** in pediatric (age 6 months and older), adult and elderly patients due to *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

### CONTRAINDICATIONS

CIPRODEX® Otic is contraindicated in patients with a history of hypersensitivity to ciprofloxacin, to other quinolones, or to any of the components in this medication. Use of this product is contraindicated in viral infections of the external canal including herpes simplex infections.

### WARNINGS

#### **FOR OTIC USE ONLY**

(This product is not approved for ophthalmic use.)

#### **NOT FOR INJECTION**

CIPRODEX® Otic should be discontinued at the first appearance of a skin rash or any other sign of hypersensitivity. Serious and occasionally fatal hypersensitivity (anaphylactic) reactions, some following the first dose, have been reported in patients receiving systemic quinolones. Serious acute hypersensitivity reactions may require immediate emergency treatment.

### PRECAUTIONS

## General

As with other antibacterial preparations, use of this product may result in overgrowth of nonsusceptible organisms, including yeast and fungi. If the infection is not improved after one week of treatment, cultures should be obtained to guide further treatment. If otorrhea persists after a full course of therapy, or if two or more episodes of otorrhea occur within six months, further evaluation is recommended to exclude an underlying condition such as cholesteatoma, foreign body, or a tumor.

The systemic administration of quinolones, including ciprofloxacin at doses much higher than given or absorbed by the otic route, has led to lesions or erosions of the cartilage in weight-bearing joints and other signs of arthropathy in immature animals of various species.

Guinea pigs dosed in the middle ear with CIPRODEX® Otic for one month exhibited no drug-related structural or functional changes of the cochlear hair cells and no lesions in the ossicles. CIPRODEX® Otic was also shown to lack dermal sensitizing potential in the guinea pig when tested according to the method of Buehler.

No signs of local irritation were found when CIPRODEX® Otic was applied topically in the rabbit eye.

## Information for Patients

For otic use only. (This product is not approved for use in the eye.) Warm the bottle in your hand for one to two minutes prior to use and shake well immediately before using.

Avoid contaminating the tip with material from the ear, fingers, or other sources.

Protect from light.

If rash or allergic reaction occurs, discontinue use immediately and contact your physician.

It is very important to use the ear drops for as long as the doctor has instructed, **even if the symptoms improve.**

Discard unused portion after therapy is completed.

**Acute Otitis Media in pediatric patients with tympanostomy tubes** Prior to administration of CIPRODEX® Otic in patients (6 months and older) with acute otitis media through tympanostomy tubes, the suspension should be warmed by holding the bottle in the hand for one or two minutes to avoid dizziness which may result from the instillation of a cold suspension. The patient should lie with the affected ear upward, and then the drops should be instilled. The tragus should then be pumped 5 times by pushing inward to facilitate penetration of the drops into the middle ear. This position should be maintained for 60 seconds. Repeat, if necessary, for the opposite ear (see **DOSAGE AND ADMINISTRATION**).

## Acute Otitis Externa

Prior to administration of CIPRODEX® Otic in patients with acute otitis externa, the suspension should be warmed by holding the bottle in the hand for one or two minutes to avoid dizziness which may result from the instillation of a cold suspension. The patient should lie with the affected ear upward, and then the drops should be instilled. This position should be maintained for 60 seconds to facilitate penetration of the drops into the ear canal. Repeat, if necessary, for the opposite ear (see **DOSAGE AND ADMINISTRATION**).

## Drug Interactions

Specific drug interaction studies have not been conducted with CIPRODEX® Otic.

## Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term carcinogenicity studies in mice and rats have been completed for ciprofloxacin. After daily oral doses of 750 mg/kg (mice) and 250 mg/kg (rats) were administered for up to 2 years, there was no evidence that ciprofloxacin had any carcinogenic or tumorigenic effects in these species. No long term studies of CIPRODEX® Otic have been performed to evaluate carcinogenic potential.

Eight *in vitro* mutagenicity tests have been conducted with ciprofloxacin, and the test results are listed below:

*Salmonella*/Microsome Test (Negative)

*E. coli* DNA Repair Assay (Negative)

Mouse Lymphoma Cell Forward Mutation Assay (Positive)

Chinese Hamster V79 Cell HGPRT Test (Negative)

Syrian Hamster Embryo Cell Transformation Assay (Negative)

*Saccharomyces cerevisiae* Point Mutation Assay (Negative)

*Saccharomyces cerevisiae* Mitotic Crossover and Gene Conversion Assay (Negative)

Rat Hepatocyte DNA Repair Assay (Positive)

Thus, 2 of the 8 tests were positive, but results of the following 3 *in vivo* test systems gave negative results:

Rat Hepatocyte DNA Repair Assay

Micronucleus Test (Mice)

### Dominant Lethal Test (Mice)

Fertility studies performed in rats at oral doses of ciprofloxacin up to 100 mg/kg/day revealed no evidence of impairment. This would be over 100 times the maximum recommended clinical dose of ototopical ciprofloxacin based upon body surface area, assuming total absorption of ciprofloxacin from the ear of a patient treated with CIPRODEX® Otic twice per day according to label directions.

Long term studies have not been performed to evaluate the carcinogenic potential of topical otic dexamethasone. Dexamethasone has been tested for *in vitro* and *in vivo* genotoxic potential and shown to be positive in the following assays; chromosomal aberrations, sister-chromatid exchange in human lymphocytes and micronuclei and sister-chromatid exchanges in mouse bone marrow. However, the Ames/Salmonella assay, both with and without S9 mix, did not show any increase in His<sup>+</sup> revertants.

The effect of dexamethasone on fertility has not been investigated following topical otic application. However, the lowest toxic dose of dexamethasone identified following topical dermal application was 1.802 mg/kg in a 26-week study in male rats and resulted in changes to the testes, epididymis, sperm duct, prostate, seminal vesicle, Cowper's gland and accessory glands. The relevance of this study for short term topical otic use is unknown.

### Pregnancy

#### Teratogenic Effects

**Pregnancy Category C:** Reproduction studies have been performed in rats and mice using oral doses of up to 100 mg/kg and IV doses up to 30 mg/kg and have revealed no evidence of harm to the fetus as a result of ciprofloxacin. In rabbits, ciprofloxacin (30 and 100 mg/kg orally) produced gastrointestinal disturbances resulting in maternal weight loss and an increased incidence of abortion, but no teratogenicity was observed at either dose. After intravenous administration of doses up to 20 mg/kg, no maternal toxicity was produced in the rabbit, and no embryotoxicity or teratogenicity was observed.

Corticosteroids are generally teratogenic in laboratory animals when administered systemically at relatively low dosage levels. The more potent corticosteroids have been shown to be teratogenic after dermal application in laboratory animals.

Animal reproduction studies have not been conducted with CIPRODEX® Otic. No adequate and well controlled studies have been performed in pregnant women. Caution should be exercised when CIPRODEX® Otic is used by a pregnant woman.

### Nursing Mothers

Ciprofloxacin and corticosteroids, as a class, appear in milk following oral administration. Dexamethasone in breast milk could suppress growth, interfere with endogenous corticosteroid production, or cause other untoward effects. It is not known whether topical otic administration of ciprofloxacin or dexamethasone could result in sufficient systemic absorption to produce detectable quantities in human milk. Because of the potential for unwanted effects in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

### Pediatric Use

The safety and efficacy of CIPRODEX® Otic have been established in pediatric patients 6 months and older (937 patients) in adequate and well-controlled clinical trials. Although no data are available on patients less than age 6 months, there are no known safety concerns or differences in the disease process in this population that would preclude use of this product. (See **DOSAGE AND ADMINISTRATION**.)

No clinically relevant changes in hearing function were observed in 69 pediatric patients (age 4 to 12 years) treated with CIPRODEX® Otic and tested for audiometric parameters.

### ADVERSE REACTIONS

In Phases II and III clinical trials, a total of 937 patients were treated with CIPRODEX® Otic. This included 400 patients with acute otitis media with tympanostomy tubes and 537 patients with acute otitis externa. The reported treatment-related adverse events are listed below:

#### Acute Otitis Media in pediatric patients with tympanostomy tubes

The following treatment-related adverse events occurred in 0.5% or more of the patients with non-intact tympanic membranes.

Adverse Event	Incidence (N=400)
Ear discomfort	3.0%



Adverse Event	Incidence (N=400)
Ear pain	2.3%
Ear precipitate (residue)	0.5%
Irritability	0.5%
Taste perversion	0.5%

The following treatment-related adverse events were each reported in a single patient: tympanostomy tube blockage; ear pruritus; tinnitus; oral moniliasis; crying; dizziness; and erythema.

#### **Acute Otitis Externa**

The following treatment-related adverse events occurred in 0.4% or more of the patients with intact tympanic membranes.

Adverse Event	Incidence (N=537)
Ear pruritus	1.5%
Ear debris	0.6%
Superimposed ear infection	0.6%
Ear congestion	0.4%
Ear pain	0.4%
Erythema	0.4%

The following treatment-related adverse events occurred in 0.4% or more of the patients with intact tympanic membranes. The following treatment-related adverse events were each reported in a single patient: ear discomfort; decreased hearing; and ear disorder (tingling).

## **DOSAGE AND ADMINISTRATION**

### **CIPRODEX® OTIC SHOULD BE SHAKEN WELL IMMEDIATELY BEFORE USE**

CIPRODEX® Otic contains 3 mg/mL (3000 µg/mL) ciprofloxacin and 1 mg/mL dexamethasone.

**Acute Otitis Media in pediatric patients with tympanostomy tubes:** The recommended dosage regimen for the treatment of acute otitis media in pediatric patients (age 6 months and older) through tympanostomy tubes is:

Four drops (0.14 mL, 0.42 mg ciprofloxacin, 0.14 mg dexamethasone) instilled into the affected ear twice daily for seven days. The suspension should be warmed by holding the bottle in the hand for one or two minutes to avoid dizziness, which may result from the instillation of a cold suspension. The patient should lie with the affected ear upward, and then the drops should be instilled. The tragus should then be pumped 5 times by pushing inward to facilitate penetration of the drops into the middle ear. This position should be maintained for 60 seconds. Repeat, if necessary, for the opposite ear. Discard unused portion after therapy is completed.

**Acute Otitis Externa:** The recommended dosage regimen for the treatment of acute otitis externa is: For patients (age 6 months and older): Four drops (0.14 mL, 0.42 mg ciprofloxacin, 0.14 mg dexamethasone) instilled into the affected ear twice daily for seven days. The suspension should be warmed by holding the bottle in the hand for one or two minutes to avoid dizziness, which may result from the instillation of a cold suspension. The patient should lie with the affected ear upward, and then the drops should be instilled. This position should be maintained for 60 seconds to facilitate penetration of the drops into the ear canal. Repeat, if necessary, for the opposite ear. Discard unused portion after therapy is completed.

## **HOW SUPPLIED**

CIPRODEX® (ciprofloxacin 0.3% and dexamethasone 0.1%) Sterile Otic Suspension is supplied as follows: 7.5 mL fill in a DROP-TAINER® system. The DROP-TAINER® system consists of a natural polyethylene bottle and natural plug, with a white polypropylene closure. Tamper evidence is provided with a shrink band around the closure and neck area of the package.

NDC 0065-8533-02, 7.5 mL fill

### **Storage**

Store at controlled room temperature, 15°C to 30°C (59°F to 86°F). Avoid freezing. Protect from light.



## Clinical Studies

In a randomized, multicenter, controlled clinical trial, CIPRODEX® Otic dosed 2 times per day for 7 days demonstrated clinical cures in the per protocol analysis in 86% of AOMT patients compared to 79% for ofloxacin solution, 0.3%, dosed 2 times per day for 10 days. Among culture positive patients, clinical cures were 90% for CIPRODEX® Otic compared to 79% for ofloxacin solution, 0.3%. Microbiological eradication rates for these patients in the same clinical trial were 91% for CIPRODEX® Otic compared to 82% for ofloxacin solution, 0.3%. In 2 randomized multicenter, controlled clinical trials, CIPRODEX® Otic dosed 2 times per day for 7 days demonstrated clinical cures in 87% and 94% of per protocol evaluable AOE patients, respectively, compared to 84% and 89%, respectively, for otic suspension containing neomycin 0.35%, polymyxin B 10,000 IU/mL, and hydrocortisone 1.0% (neo/poly/HC). Among culture positive patients clinical cures were 86% and 92% for CIPRODEX® Otic compared to 84% and 89%, respectively, for neo/poly/HC. Microbiological eradication rates for these patients in the same clinical trials were 86% and 92% for CIPRODEX® Otic compared to 85% and 85%, respectively, for neo/poly/HC.

## References

1. Campoli-Richards DM, Monk JP, Price A, Benfield P, Todd PA, Ward A. Ciprofloxacin: A review of its antibacterial activity, pharmacokinetic properties and therapeutic use. *Drugs* 1988;35:373-447.
2. Loew D, Schuster O, and Graul E. Dose-dependent pharmacokinetics of dexamethasone. *Eur J Clin Pharmacol* 1986;30:225-230.

U.S. Patent Nos. 4,844,902; 6,284,804; 6,359,016  
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Manufactured by Alcon Laboratories, Inc.  
Rx Only  
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Revision date: 17 July 2003

## PATIENT INFORMATION

CIPRODEX® (CI-PRO-DEX)  
(ciprofloxacin 0.3% and dexamethasone 0.1%)  
Sterile Otic Suspension

## IMPORTANT PATIENT INFORMATION AND INSTRUCTIONS. READ BEFORE USE.

### What is CIPRODEX® Otic?

CIPRODEX® Otic is an antibiotic/steroid combination product in a sterile suspension used to treat:

- **Middle Ear Infection with Drainage Through a Tube in Children 6 months and older:** A middle ear infection is a bacterial infection behind the eardrum. People with a tube in the eardrum may notice drainage from the ear canal.
- **Outer Ear Canal Infection in Patients 6 months and older:** An outer ear canal infection, also known as "Swimmer's Ear", is a bacterial infection of the outer ear canal. The ear canal and the outer part of the ear may swell, turn red, and be painful. Also, a fluid discharge may appear in the ear canal.

### Who should NOT use CIPRODEX® Otic?

- Do not use this product if allergic to ciprofloxacin or to other quinolone antibiotics.
- Do not use this product if allergic to dexamethasone or to other steroids.
- Do not give this product to pediatric patients who are less than 6 months old.

### How often should CIPRODEX® Otic be given?

CIPRODEX® Otic ear drops should be given 2 times each day (about 12 hours apart, for example, 8 AM and 8 PM) in each infected ear unless the doctor has instructed otherwise. The best times to use the ear drops are in the morning and at night. It is very important to use the ear drops for as long as the doctor has instructed, **even if the symptoms improve**. If CIPRODEX® Otic ear drops are not used for as long as the doctor has instructed, the infection may return.

### What if a dose is missed?

If a dose of CIPRODEX® Otic is missed, it should be given as soon as possible. If it is almost time for the next dose, skip the missed dose and go back to the regular dosing schedule. Do not use a double dose unless the doctor has instructed you to do so. If the infection is not improved after one week, you should consult your doctor. If you have two or more episodes of drainage within six months, it is recommended you see

your doctor for further evaluation.

**What activities should be avoided while using CIPRODEX® Otic?**

It is important that the infected ear(s) remain clean and dry. When bathing, avoid getting the infected ear(s) wet. Avoid swimming unless the doctor has instructed otherwise.

**What are the possible side effects of CIPRODEX® Otic?**

During the testing of CIPRODEX® Otic for middle ear infections, the most common side effect related to CIPRODEX® Otic was ear discomfort that occurred in up to 3 out of 100 patients. Other common side effects were: ear pain; ear precipitate (residue); irritability; and abnormal taste. During the testing of CIPRODEX® Otic for ear canal infections, the most common side effect related to CIPRODEX® Otic was itching of the ear that occurred in 1 to 2 out of 100 patients. Other common side effects were: ear debris; ear infection in the treated ear; ear congestion; ear pain; and rash.

If any of these side effects persist, call the doctor.

If an allergic reaction to CIPRODEX® Otic occurs, stop using the product and contact your doctor.

**DO NOT TAKE BY MOUTH**

If CIPRODEX® Otic is accidentally swallowed or overdose occurs, call the doctor immediately. This medicine is available only with a doctor's prescription. Use only as directed. Do not use this medicine if outdated. If you wish to learn more about CIPRODEX® Otic, call your doctor or pharmacist.

**HOW SUPPLIED**

CIPRODEX® Otic is supplied as follows: 7.5 mL fill in a DROP-TAINER® system. The DROP-TAINER® system consists of a natural polyethylene bottle and natural plug, with a white polypropylene closure. Tamper evidence is provided with a shrink band around the closure and neck area of the package.  
NDC 0065-8533-02, 7.5 mL fill

**Storage**

Store at controlled room temperature, 15°C to 30°C (59°F to 86°F). Avoid freezing. Protect from light.

U.S. Patent Nos. 4,844,902; 6,284,804; 6,359,016

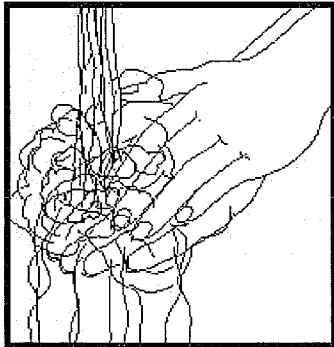
CIPRODEX® is a registered trademark of Bayer AG.

Licensed to Alcon, Inc. by Bayer AG.

Manufactured by Alcon Laboratories, Inc.

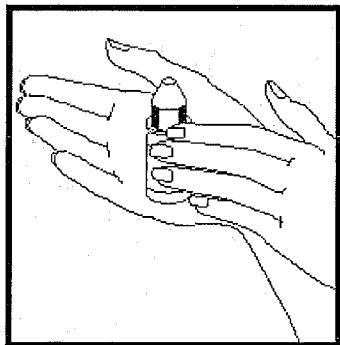
Rx Only

©2003, 2004 Alcon, Inc.

**How should CIPRODEX® Otic be given?****1. Wash hands**

The person giving CIPRODEX® Otic should wash his/her hands with soap and water.

**2. Warm & shake bottle**

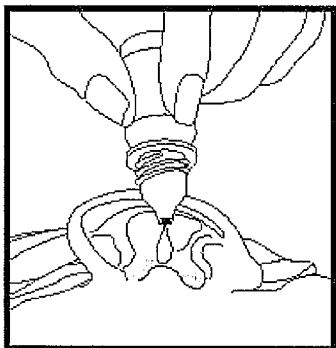


Hold the bottle of CIPRODEX® Otic in the hand for one or two minutes to warm the suspension, then shake well.

### 3. Add drops



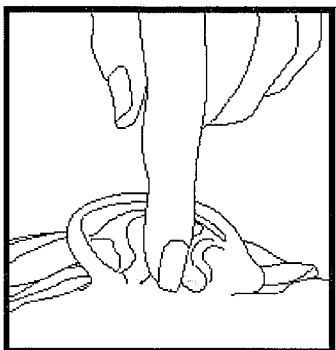
The person receiving CIPRODEX® Otic should lie on his/her side with the infected ear up.



Patients should have 4 drops of CIPRODEX® Otic put into the infected ear. The tip of the bottle should not touch the fingers, or the ear, or any other surfaces.

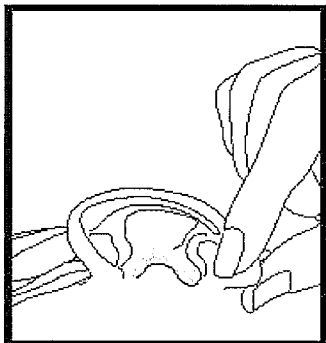
**BE SURE TO FOLLOW INSTRUCTIONS BELOW FOR THE PATIENT'S SPECIFIC EAR INFECTION.**

### 4. For Patients with Middle Ear Infection with Tubes:



While the person receiving CIPRODEX® Otic lies on his/her side, the person giving the drops should gently press the tragus (see diagram) 5 times in a pumping motion. This will allow the drops to pass through the tube in the eardrum and into the middle ear.

**5. For Patients with Outer Ear Infection ("Swimmer's Ear"):**



While the person receiving the drops lies on his/her side, the person giving the drops should gently pull the outer ear lobe upward and backward. This will allow the ear drops to flow down into the ear canal.

**6. Stay on side**



The person who received the ear drops should remain on his/her side for at least 60 seconds.

Repeat Steps 2-5 for the other ear if both ears are infected.

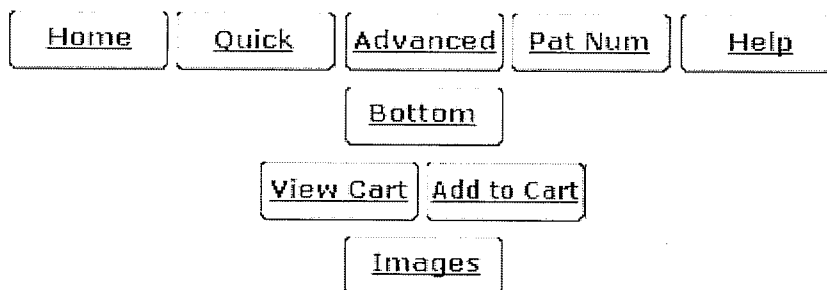
<b>CIPRODEX</b>			
ciprofloxacin and dexamethasone suspension			
<b>Product Information</b>			
<b>Product Type</b>	HUMAN PRESCRIPTION DRUG	<b>NDC Product Code (Source)</b>	0065-8533
<b>Route of Administration</b>	AURICULAR (OTIC)	<b>DEA Schedule</b>	
<b>INGREDIENTS</b>			
Name (Active Moiety)	Type	Strength	
ciprofloxacin hydrochloride (ciprofloxacin)	Active	3 MILLIGRAM In 1 MILLILITER	
dexamethasone (dexamethasone)	Active	1 MILLIGRAM In 1 MILLILITER	
benzalkonium chloride	Inactive	0.1 MILLIGRAM In 1 MILLILITER	
boric acid	Inactive		
sodium chloride	Inactive		
hydroxyethyl cellulose	Inactive		
tyloxapol	Inactive		
acetic acid	Inactive		
sodium acetate	Inactive		
edetate disodium	Inactive		
water	Inactive		

sodium hydroxide and/or hydrochloric acid		Inactive
<b>Product Characteristics</b>		
Color		Score
Shape		Size
Flavor		Imprint Code
Contains		
<b>Packaging</b>		
# NDC	Package Description	Multilevel Packaging
1 0065-8533-01	5 mL (MILLILITER) In 1 BOTTLE, PLASTIC	None
2 0065-8533-02	7.5 mL (MILLILITER) In 1 BOTTLE, PLASTIC	None
3 0065-8533-03	1.5 mL (MILLILITER) In 1 BOTTLE, PLASTIC	None
4 0065-8533-04	7.5 mL (MILLILITER) In 1 BOTTLE, PLASTIC	None

Revised: 07/2007

Alcon, Inc.

EXHIBIT F  
PATENT 4,357,324

USPTO PATENT FULL-TEXT AND IMAGE DATABASE

( 1 of 1 )

**United States Patent**  
**Montgomery , et al.**

**4,357,324**  
**November 2, 1982**

Prodrug derivatives of 9.beta.-D-arabinofuranosyl-2-fluoroadenine

**Abstract**

The 5'-formate and the 5'-phosphate derivatives of 9-.beta.-D-arabinofuranosyl-2-fluoroadenine have been prepared as prodrug forms of the anti-cancer agent 9-.beta.-D-arabinofuranosyl-2-fluoroadenine, known as F-ara-A. These derivatives are quite water soluble whereas F-ara-A itself is sparingly soluble in water or in any organic solvents. Delivery of these prodrug forms to mice with L1210 leukemia results in the formation of higher levels of the triphosphate of F-ara-A, the active form of the drug, in the target L1210 leukemia cells. These prodrug forms are much more active chemotherapeutically than 9-.beta.-D-arabinofuranosyladenine, known as ara-A, and equivalent in activity to the combination of ara-A and 2'-deoxycoformycin, known as 2'-dCF, an effective in vivo inhibitor of adenosine deaminase, a ubiquitous enzyme that destroys ara-A in vivo.

Inventors: **Montgomery; John A.** (Birmingham, AL), **Shortnacy; Anita T.** (Birmingham, AL)  
 Assignee: **The United States of America as represented by the Department of health** (Washington, DC)  
 Appl. No.: **06/237,617**  
 Filed: **February 24, 1981**

**Current U.S. Class:** 514/45 ; 514/48; 536/26.7; 536/27.4; 536/27.63; 536/27.7  
**Current International Class:** C07H 19/16 (20060101); C07H 19/00 (20060101); A61K 031/70 (); C07H 019/18 (); C07H 019/20 ()  
**Field of Search:** 536/26,27 424/180

**References Cited [Referenced By]****U.S. Patent Documents**

<u>3703507</u>	November 1972	Haskell et al.
<u>4093714</u>	June 1978	Tolman et al.

<u>4123609</u>	October 1978	Behnke et al.
<u>4136175</u>	January 1979	Rideout et al.
<u>4188378</u>	February 1980	Montgomery
<u>4210745</u>	July 1980	Montgomery

### Other References

Repta et al., "Jour. Phar. Science", 64 pp. 392-396, 1975..

*Primary Examiner:* Brown; Johnnie R.

*Attorney, Agent or Firm:* Roberts, Jr.; John S.

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### Government Interests

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The invention described herein was made in the course of work under a grant or award from the Department of Health and Human Services.

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### Claims

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We claim:

1. 9-(5-O-formyl-.beta.-D-arabinofuranosyl)-2-fluoroadenine.
2. A method for treating L1210 cancer in mice by administration of an aqueous solution of an effective amount of an agent consisting of 5'-O-formyl derivative of O-.beta.-D-arabinofuranosyl-2-fluoroadenine.
3. A method of treating mouse cancer according to claim 2 wherein an oral dosage is administered in an effective amount of a dosage of 10 to 250 mg/kg/day in a unitary regimen.
4. 9-(5-O-phosphate-.beta.-D-arabinofuranosyl)-2-fluoroadenine.
5. A method of treating L1210 mouse cancer by administration of an aqueous solution of an effective amount of an agent consisting of 5-O-phosphate derivative of O-.beta.-D-arabinofuranosyl-2-fluoroadenine.
6. A method of treating mouse cancer by administration of an effective amount of an aqueous solution of 5' phosphate of 9-.beta.-D-arabinofuranosyl-2-fluoroadenine.
7. A method for treating L1210 cancer in mice wherein the 5' phosphate of 9-.beta.-D-arabinofuranosyl-2-fluoroadenine is administered in an effective amount of a dosage of 10 to 250 mg.

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### Description

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## SUMMARY AND DETAILED DESCRIPTION

This invention relates to preparation and utilization in the treatment of leukemia L1210 in mice of new compounds that serve as prodrug forms of the anticancer agent 9-.beta.-D-arabinofuranosyl-2-fluoroadenine, known as F-ara-A. These compounds, the 5'-formate and the 5'-phosphate (F-ara-AMP) of F-ara-A, are water-soluble, chemotherapeutically effective forms of the drug that are converted to the parent drug in vivo. The parent drug is phosphorylated in the target L1210 Leukemia to the triphosphate which causes cell death and, hence, therapeutic activity against the disease. The data in Table I clearly show that the levels of F-ara-ATP in L1210 resulting from near equimolar doses of F-ara, its 5'-formate, F-ara-AMP, and ara-A Plus 2'-dCF are significantly higher from the prodrug forms of F-ara-A than from F-ara-A itself which in turn results in higher levels of the triphosphate than ara-A+2'dCF. These higher levels of the active agent that last for longer periods of time allow effective treatment with these drugs given once a day for nine days (see FIGS. 1, 2 and 3). In contrast, ara-A on its optimal schedule-every 3 hours for 24 hours-is not very effective and even given with 2'-dCF is no better than the prodrug forms of F-ara-A. Thus the properties of these prodrugs will allow them to be used effectively given intravenously once a day as opposed to a constant infusion of a two-drug combination, the second component of which has clear but not completely defined toxicity.

TABLE I	Treatment Nanomoles of Dose Time After											
Triphosphate per Agent (mmole/kg)	Treatment gm L1210 cells											
	F-ara-A	1.4	3	220	0.5	3	160	F-ara-A5'	1.2	3	400	
formate	0.6	3	125	F-are-AMP	0.7	2	220	are-AMP (+2'dCF)	0.6	2	110	3 69

For use as anti-cancer agents, the prodrug of this invention or their salts may be given parenterally (in an injectable solution), orally (tablets or capsules), used as a suppository, applied as an ophthalmic solution, or applied topically as an ointment, cream, powder, etc., as a pharmaceutical preparation in accordance with known medical or veterinarial practice. The preferred oral dosage is administered at a dosage of about 10 to 250 mg/kg of mammal body weight (i.e. mice, rats). A controlling dosage of not greater than half the LD 50 is maintained and required.

## PRIOR ART STATEMENT

1. U.S. Pat. No. 3,703,507 Haskell/Watson, shows the 9-(Beta-d-arabinofuranosyl) adenine, 5' phosphate.
2. Repta, et al., J. Pharm. Sci., 64 392 (1975) This article teaches the 5' formate of the basic compound F-ara-A.
3. U.S. Pat. No. 4,093,714 Tolman, et al., teaches the 5' phosphorylated 9-.beta.-D-arabinofuranoside.
4. U.S. Pat. No. 4,136,175 Rideout (Burroughs Wellcome Co.) and U.S. Pat. No. 4,123,609 Behnke (Warner-Lambert Co.) both disclose 9-.beta.-D-arabinofuranosyl adenine 5'-phosphate.

## DESCRIPTION OF DRAWINGS

FIG. 1 shows the comparison of activity of 5-formyl-2-F-ara-A and 2'-dCF plus ara-A against L1210/0 Leukemia as further explained in Table II.

FIG. 2 shows the activity of ara-A plus 2'-dCF against L1210/0 leukemia as further explained in Table III.

FIG. 3 shows the comparison of 2-fluoro-ara-AMP and 2'-dCF plus ara-A against L1210 leukemia 10.sup.5 L1210 cell IP as further explained in Table IV.

TABLE II Comparison of Activity of 5-Formyl-2-F-ara-A and 2'dCF Plus Ara-A Against L1210 Treatment: IP; QD 1-9 Days\* Dosage Median 10.sup.5 IP (MG/KG/ Lifespan, % ILS L1210/0 Dose) Days (Cures)

Control 9 -- (untreated)	5-Formyl-2-	150	25	177	(3/10)	F-ara-A 2'dCF 0.25	++	13.5	50	ara-A 150	2'dCF 0.25	++	13.5	50	ara-AMP 315	*2'dCF Given 30 Minutes Before araA
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TABLE III Activity of ara-A Plus 2'dCF Against L1210/0 Leukemia Treatment: IP Dosage Median 10.sup.5 IP (MG/KG/ Lifespan, % ILS L1210/0 Dose) Days (Cures)

Control 9 -- (Untreated)	Ara-A 200	++	14	55	2'dCF 0.25	ara-A 54	++	22	144	(3/10)	2'dCF 0.2
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TABLE IV Comparison of 2-Fluoro-Ara-AMP and 2'dCF Plus Ara-A Against L1210 Leukemia 10.sup.5 L1210 Cell IP Dosage Lifespan % ILS Compound mg/kg/day Schedule Days (Cures)

Control (untreated)	8
2-Fluoro- ara-AMP 219	qd 1-9 18 125(3/10)
Ara-AMP 375	q3h,days 1,5,9 13 62
Ara-AMP 125	+ q3h,days 1,5,9 22.5 181(4/10)
2'dCf 0.05	

Preparation of Prodrug Forms of 9-.beta.-D-arabinofuranosyl-2-fluoroadenine ##STR1##

The 5'-formyl derivative (R.dbd.CHO) of F-ara-A was prepared by the reaction for F-ara-A with cold 98% formic acid. It was isolated by acetone extraction followed by reverse-phase high-pressure liquid chromatography on a C.sub.18 using water-acetonitrile as eluant.

The 5'-phosphate (R.dbd.H.sub.2 PO.sub.3 --) of F-ara-A was prepared by the reaction of F-ara-A with phosphorous oxychloride in an alkyl phosphate followed by hydrolysis in water. The product was purified by adsorption on and elution from a mixture of charcoal and Celite.

#### The Antitumor Activity

It has been noted that the antitumor activity of 9-.beta.-D-arabinofuranosyl adenine (ara-A) is enhanced by the incorporation of a fluorine atom at the 2-carbon. This structural feature presents the enzymatic destruction of ara-A by the ubiquitous enzyme adenosine deaminase but does not interfere with phosphorylation of the molecule and destruction of leukemic cells by the resulting triphosphate of F-ara-A, however, it is very insoluble and difficult to administer properly. To overcome this difficulty two water-soluble derivatives, the 5'-formate and the 5'-phosphate, were prepared. These compounds provided higher levels of F-ara-A triphosphate in leukemic cells than F-ara-A itself and resulted in good increases in lifespan and some cures of mice implanted with leukemia L1210 cells (see FIGS. 1 and 3). The 5'-formyl derivative gave a 177% increase in lifespan and three cures.

#### EXAMPLE I

##### 9-(5-O-formyl-.beta.-D-arabinofuranosyl)-2-fluoroadenine

A solution of 9-.beta.-D-arabinofuranosyl-2-fluoroadenine (3.3 g, 11.6 mmol) in 98% formic acid (17.5 ml) was allowed to stand at 4.degree. for seven days before it was evaporated to dryness in vacuo

without heat. Cold heptane was added to the residue and evaporated in vacuo twice followed by cold acetone. The white solid was extracted with hot acetone, and the acetone solution evaporated to dryness in vacuo; yield, 3.22 g. High-pressure liquid chromatography and pmr data showed this material to be a mixture of 9-(5-O-formyl-.beta.-D-arabinofuranosyl)-2-fluoroadenine (65%), 9-(3,5-di-O-formyl-.beta.-D-arabinofuranosyl)-2-fluoroadenine (20%), 9-(2,5-di-O-formyl-.beta.-D-arabinofuranosyl)-2-fluoroadenine (9%), and 9-.beta.-D-arabinofuranosyl-2-fluoroadenine (6%). These compounds were separated by preparative high-pressure liquid chromatography using a C.sub.18 column eluted with water: acetonitrile (17:3). 5'-Formate-pmr in S: 4:15 (m, H.sub.2, H.sub.3 ', and H.sub.4 '), 4.4 (m, 2H.sub.5 '), 5.8 (broad, OH), 6.2 (d, J.sub.1 '.sub.2 ', 4 Hz, H.sub.1 '), 7.8 (s, NH.sub.2), 8.1 (s, H.sub.8), 8.3 (s, H of CHO). 3', 5'-Diformate-pmr in S: 4.2 (m, H.sub.2 ' and H.sub.4 '), 5.4 (t, H.sub.3 '), 6.23 (d, J.sub.1 '.sub.2 ', 4 Hz, H.sub.1 '), 7.85 (s, NH.sub.2), 8.15 (s, H.sub.8), 8.3 and 8.4 (2s, 2 CHO).

## EXAMPLE II

9-.beta.-D-arabinofuranosyl-2-fluoroadenine 5'-phosphate (R.dbd.H.sub.2 PO.sub.3 --)

9-.beta.-D-Arabinofuranosyl-2-fluoroadenine (2.6 g, 9.1 mmol) was added to 23 ml of triethylphosphate containing POCl.sub.3 (4.6 g, 30 mmol) held at 0.degree., and the mixture was stirred for 3 1/2 hours before it was poured into 200 ml of ice water. The pH of the solution was adjusted to 2 with 6 N NaOH before it was extracted with CHCl.sub.3 (2.times.180 ml) and then slurried with charcoal (40 g) and Celite (20 g) for 20 min. The solids were removed by filtration and washed with water until free of acid. The product was extracted with a mixture of SOEtOH:NH.sub.2 OH:9H.sub.2 O. The basic solution was lyophilized to give the product 3.46 g.

Some of this material was converted to the free acid by means of a Domex 1.times.8 (formate form) column. UV.sub.max (e.times.10.sup.-3): 262 (13.5), pH 7-262 (15.5), pH 13-261 (15.2).

Anal. Calcd. for C.sub.10 H.sub.13 FN.sub.5 O.sub.7 P.3/4H.sub.2 O: C, 31.71; H, 3.86; N, 18.49. Found: C, 32.00; H, 3.86; N, 18.35.

\* \* \* \* \*

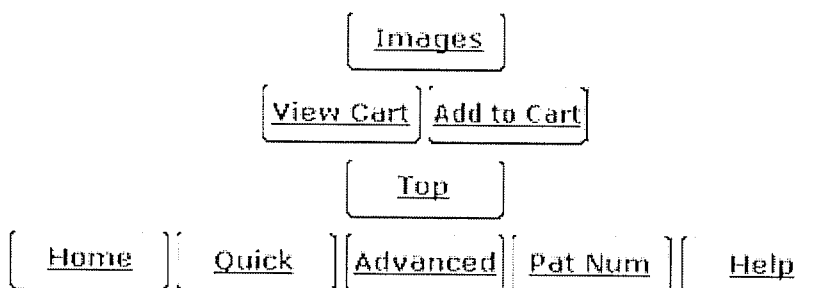


EXHIBIT G  
FLUDARA<sup>®</sup> LABEL

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Version 3 - Published Apr 21, 2009

**FLUDARA - fludarabine phosphate injection, powder, lyophilized, for solution**  
Bayer HealthCare Pharmaceuticals Inc.

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**Fludara**  
**(fludarabine phosphate)**

**FOR INJECTION**

**FOR INTRAVENOUS USE ONLY**

**Rx Only**

#### WARNING

FLUDARA FOR INJECTION should be administered under the supervision of a qualified physician experienced in the use of antineoplastic therapy. FLUDARA FOR INJECTION can severely suppress bone marrow function. When used at high doses in dose-ranging studies in patients with acute leukemia, FLUDARA FOR INJECTION was associated with severe neurologic effects, including blindness, coma, and death. This severe central nervous system toxicity occurred in 36% of patients treated with doses approximately four times greater (96 mg/m<sup>2</sup>/day for 5-7 days) than the recommended dose. Similar severe central nervous system toxicity, including coma, seizures, agitation and confusion, has been reported in patients treated at doses in the range of the dose recommended for chronic lymphocytic leukemia.

Instances of life-threatening and sometimes fatal autoimmune phenomena such as hemolytic anemia, autoimmune thrombocytopenia/thrombocytopenic purpura (ITP), Evan's syndrome, and acquired hemophilia have been reported to occur after one or more cycles of treatment with FLUDARA FOR INJECTION. Patients undergoing treatment with FLUDARA FOR INJECTION should be evaluated and closely monitored for hemolysis.

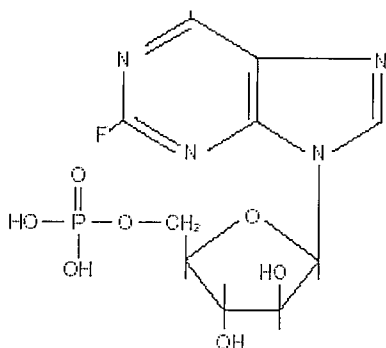
In a clinical investigation using FLUDARA FOR INJECTION in combination with pentostatin (deoxycoformycin) for the treatment of refractory chronic lymphocytic leukemia (CLL), there was an unacceptably high incidence of fatal pulmonary toxicity. Therefore, the use of FLUDARA FOR INJECTION in combination with pentostatin is not recommended.

#### DESCRIPTION

FLUDARA FOR INJECTION contains fludarabine phosphate, a fluorinated nucleotide analog of the antiviral agent vidarabine, 9-β-D-arabinofuranosyladenine (ara-A) that is relatively resistant to deamination by adenosine deaminase. Each vial of sterile lyophilized solid cake contains 50 mg of the active ingredient fludarabine phosphate, 50 mg of mannitol, and sodium hydroxide to adjust pH to 7.7. The pH range for the final product is 7.2-8.2. Reconstitution with 2 mL of Sterile Water for Injection USP results in a solution containing 25 mg/mL of fludarabine phosphate intended for intravenous administration.

The chemical name for fludarabine phosphate is 9H-Purin-6-amine, 2-fluoro-9-(5-0-phosphono-β-D-arabino-furanosyl) (2-fluoro-ara-AMP). The molecular formula of fludarabine phosphate is C<sub>10</sub>H<sub>13</sub>FN<sub>5</sub>O<sub>7</sub>P (MW 365.2) and the structure is:





## CLINICAL PHARMACOLOGY

Fludarabine phosphate is rapidly dephosphorylated to 2-fluoro-ara-A and then phosphorylated intracellularly by deoxycytidine kinase to the active triphosphate, 2-fluoro-ara-ATP. This metabolite appears to act by inhibiting DNA polymerase alpha, ribonucleotide reductase and DNA primase, thus inhibiting DNA synthesis. The mechanism of action of this antimetabolite is not completely characterized and may be multifaceted.

Phase I studies in humans have demonstrated that fludarabine phosphate is rapidly converted to the active metabolite, 2-fluoro-ara-A, within minutes after intravenous infusion. Consequently, clinical pharmacology studies have focused on 2-fluoro-ara-A pharmacokinetics. After the five daily doses of 25 mg 2-fluoro-ara-AMP/m<sup>2</sup> to cancer patients infused over 30 minutes, 2-fluoro-ara-A concentrations show a moderate accumulation. During a 5-day treatment schedule, 2-fluoro-ara-A plasma trough levels increased by a factor of about 2. The terminal half-life of 2-fluoro-ara-A was estimated as approximately 20 hours. *In vitro*, plasma protein binding of fludarabine ranged between 19% and 29%.

A correlation was noted between the degree of absolute granulocyte count nadir and increased area under the concentration x time curve (AUC).

### Special Populations

#### *Pediatric Patients*

Limited pharmacokinetic data for FLUDARA FOR INJECTION are available from a published study of children (ages 1-21 years) with refractory acute leukemias or solid tumors (Children's Cancer Group Study 097<sup>1</sup>). When FLUDARA FOR INJECTION was administered as a loading dose over 10 minutes immediately followed by a 5-day continuous infusion, steady-state conditions were reached early.

#### *Patients with Renal Impairment*

The total body clearance of the principal metabolite 2-fluoro-ara-A correlated with the creatinine clearance, indicating the importance of the renal excretion pathway for the elimination of the drug. Renal clearance represents approximately 40% of the total body clearance. Patients with moderate renal impairment (17 - 41 mL/min/m<sup>2</sup>) receiving 20% reduced Fludara dose had a similar exposure (AUC; 21 versus 20 nM•h/mL) compared to patients with normal renal function receiving the recommended dose. The mean total body clearance was 172 mL/min for normal and 124 mL/min for patients with moderately impaired renal function.

## CLINICAL STUDIES

Two single-arm open-label studies of FLUDARA FOR INJECTION have been conducted in adult patients with CLL refractory to at least one prior standard alkylating-agent containing regimen. In a study conducted by M.D. Anderson Cancer Center (MDAH), 48 patients were treated with a dose of 22-40 mg/m<sup>2</sup> daily for 5 days every 28 days. Another study conducted by the Southwest Oncology Group (SWOG) involved 31 patients treated with a dose of 15-25 mg/m<sup>2</sup> daily for 5 days every 28 days. The overall objective response rates were 48% and 32% in the MDAH and SWOG studies, respectively. The complete response rate in both studies was 13%; the partial response rate was 35% in the MDAH study and 19% in the SWOG study. These response rates were obtained using standardized response criteria developed by the National Cancer Institute CLL Working Group<sup>3</sup> and were achieved in heavily pre-treated patients. The ability of FLUDARA FOR INJECTION to induce a significant rate of response in refractory patients suggests minimal cross-resistance with commonly used anti-CLL agents.



The median time to response in the MDAH and SWOG studies was 7 weeks (range of 1 to 68 weeks) and 21 weeks (range of 1 to 53 weeks) respectively. The median duration of disease control was 91 weeks (MDAH) and 65 weeks (SWOG). The median survival of all refractory CLL patients treated with FLUDARA FOR INJECTION was 43 weeks and 52 weeks in the MDAH and SWOG studies, respectively.

Rai stage improved to Stage II or better in 7 of 12 MDAH responders (58%) and in 5 of 7 SWOG responders (71%) who were Stage III or IV at baseline. In the combined studies, mean hemoglobin concentration improved from 9.0 g/dL at baseline to 11.8 g/dL at the time of response in a subgroup of anemic patients. Similarly, average platelet count improved from 63,500/mm<sup>3</sup> to 103,300/mm<sup>3</sup> at the time of response in a subgroup of patients who were thrombocytopenic at baseline.

## INDICATIONS AND USAGE

FLUDARA FOR INJECTION is indicated for the treatment of adult patients with B-cell chronic lymphocytic leukemia (CLL) who have not responded to or whose disease has progressed during treatment with at least one standard alkylating-agent containing regimen. The safety and effectiveness of FLUDARA FOR INJECTION in previously untreated or non-refractory patients with CLL have not been established.

## CONTRAINDICATIONS

FLUDARA FOR INJECTION is contraindicated in those patients who are hypersensitive to this drug or its components.

## WARNINGS

### (See **BOXED WARNINGS**)

There are clear dose dependent toxic effects seen with FLUDARA FOR INJECTION. Dose levels approximately 4 times greater (96 mg/m<sup>2</sup>/day for 5 to 7 days) than that recommended for CLL (25 mg/m<sup>2</sup>/day for 5 days) were associated with a syndrome characterized by delayed blindness, coma and death. Symptoms appeared from 21 to 60 days following the last dose. Thirteen of 36 patients (36%) who received FLUDARA FOR INJECTION at high doses (96 mg/m<sup>2</sup>/day for 5 to 7 days) developed this severe neurotoxicity. Similar severe central nervous system toxicity, including coma, seizures, agitation and confusion, has been reported in patients treated at doses in the range of the dose recommended for chronic lymphocytic leukemia.

The effect of chronic administration of FLUDARA FOR INJECTION on the central nervous system is unknown, however, patients have received the recommended dose for up to 15 courses of therapy.

Severe bone marrow suppression, notably anemia, thrombocytopenia and neutropenia, has been reported in patients treated with FLUDARA FOR INJECTION. In a Phase I study in adult solid tumor patients, the median time to nadir counts was 13 days (range, 3-25 days) for granulocytes and 16 days (range, 2-32) for platelets. Most patients had hematologic impairment at baseline either as a result of disease or as a result of prior myelosuppressive therapy. Cumulative myelosuppression may be seen. While chemotherapy-induced myelosuppression is often reversible, administration of FLUDARA FOR INJECTION requires careful hematologic monitoring.

Several instances of trilineage bone marrow hypoplasia or aplasia resulting in pancytopenia, sometimes resulting in death, have been reported in adult patients. The duration of clinically significant cytopenia in the reported cases has ranged from approximately 2 months to approximately 1 year. These episodes have occurred both in previously treated or untreated patients.

Instances of life-threatening and sometimes fatal autoimmune phenomena such as hemolytic anemia, autoimmune thrombocytopenia/thrombocytopenic purpura (ITP), Evan's syndrome, and acquired hemophilia have been reported to occur after one or more cycles of treatment with FLUDARA FOR INJECTION in patients with or without a previous history of autoimmune hemolytic anemia or a positive Coombs' test and who may or may not be in remission from their disease. Steroids may or may not be effective in controlling these hemolytic episodes. The majority of patients rechallenged with FLUDARA FOR INJECTION developed a recurrence in the hemolytic process. The mechanism(s) which predispose patients to the development of this complication has not been identified. Patients undergoing treatment with FLUDARA FOR INJECTION should be evaluated and closely monitored for hemolysis. Discontinuation of therapy with Fludara is recommended in case of hemolysis.

Transfusion-associated graft-versus-host disease has been observed after transfusion of non-irradiated blood in FLUDARA FOR INJECTION treated patients. Fatal outcome as a consequence of this disease has been reported. Therefore, to minimize the risk of transfusion-associated graft-versus-host disease, patients who require blood transfusion and who are undergoing, or who have received, treatment with FLUDARA FOR INJECTION should receive irradiated blood only.

In a clinical investigation using FLUDARA FOR INJECTION in combination with pentostatin (deoxycoformycin) for the treatment of refractory chronic lymphocytic leukemia (CLL) in adults, there was an unacceptably high incidence of fatal pulmonary toxicity. Therefore, the use of FLUDARA FOR INJECTION in combination with pentostatin is not recommended.

Of the 133 adult CLL patients in the two trials, there were 29 fatalities during study. Approximately 50% of the fatalities were due to infection and 25% due to progressive disease.

#### Pregnancy Category D

Based on its mechanism of action, fludarabine phosphate can cause fetal harm when administered to a pregnant woman. There are no adequate and well-controlled studies of Fludara in pregnant women. Fludarabine phosphate was embryo-lethal and teratogenic in both rats and rabbits. If FLUDARA FOR INJECTION is used during pregnancy, or if the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus. Women of childbearing potential should be advised to avoid becoming pregnant. Women of childbearing potential and fertile males must take contraceptive measures during and at least for six months after cessation of treatment with FLUDARA FOR INJECTION.

Fludarabine phosphate was embryo lethal and teratogenic in rats and rabbits.

Fludarabine phosphate was administered at doses of 0, 1, 10 or 30 mg/kg/day (0.24, 2.4 times and 7.2 times the recommended human dose on a mg/m<sup>2</sup> basis, respectively) to pregnant rats on days 6 to 15 of gestation.

At 10 and 30 mg/kg/day administered during organogenesis, there was a dose-related increase in various skeletal variations and a decrease in mean fetal body weights. Maternal toxicity was not apparent at 10 mg/kg/day, and was limited to slight body weight decreases at 30 mg/kg/day. In a dose finding study malformations, such as limb and tail defects, were induced at 40 mg/kg/day (9.6 times the recommended human dose on a mg/m<sup>2</sup> basis). In a reproduction toxicity study on rabbits Fludarabine phosphate was administered intravenously at doses of 0, 1, 5 or 8 mg/kg/day (approximately 0.5, 2.4, and 3.8 times the recommended human dose on a mg/m<sup>2</sup> basis) on days 6 to 18 of gestation. A dose of 8 mg/kg/day administered during organogenesis increased embryo and fetal lethality as indicated by a higher number of resorptions and a decrease in live fetuses. Compound-related teratogenic effects manifested by external deformities and skeletal malformations were observed at 8 mg/kg/day. The most frequent external malformations observed in rabbits were cleft palate, adactyly, brachydactyly and syndactyly along with skeletal malformations such as fused metatarsals, phalanges, sternebrae and limb bones and some soft tissue malformations (diaphragmatic herniae). Fetal body weights were decreased in rabbits given 8 mg/kg/day.

## PRECAUTIONS

### General

FLUDARA FOR INJECTION is a potent antineoplastic agent with potentially significant toxic side effects. Patients undergoing therapy should be closely observed for signs of hematologic and nonhematologic toxicity. Periodic assessment of peripheral blood counts is recommended to detect the development of anemia, neutropenia and thrombocytopenia.

Tumor lysis syndrome associated with FLUDARA FOR INJECTION treatment has been reported in CLL patients with large tumor burdens. Since FLUDARA FOR INJECTION can induce a response as early as the first week of treatment, precautions should be taken in those patients at risk of developing this complication.

In patients with impaired state of health, FLUDARA FOR INJECTION should be given with caution and after careful risk/benefit consideration. This applies especially for patients with severe impairment of bone marrow function (thrombocytopenia, anemia, and/or granulocytopenia), immunodeficiency or with a history of opportunistic infection. Prophylactic treatment should be considered in patients at increased risk of developing opportunistic infections.

There are inadequate data on dosing of patients with renal insufficiency. FLUDARA FOR INJECTION must be administered cautiously in patients with renal insufficiency. The total body clearance of 2-fluoro-ara-A has been shown to be directly correlated with creatinine clearance. Patients with moderate impairment of renal function (creatinine clearance 30-70 mL/min/1.73 m<sup>2</sup>) should have their Fludara dose reduced by 20% and be monitored closely. FLUDARA FOR INJECTION is not recommended for patients with severely impaired renal function (creatinine clearance less than 30 mL/min/1.73 m<sup>2</sup>).

Fludara may reduce the ability to drive or use machines, since fatigue, weakness, visual disturbances, confusion, agitation and seizures have been observed.

### Laboratory Tests

During treatment, the patient's hematologic profile (particularly neutrophils and platelets) should be monitored regularly to determine the degree of hematopoietic suppression.



### Drug Interactions

The use of FLUDARA FOR INJECTION in combination with pentostatin is not recommended due to the risk of severe pulmonary toxicity (see **WARNINGS** section).

### Carcinogenesis

No animal carcinogenicity studies with FLUDARA FOR INJECTION have been conducted.

### Mutagenesis

Fludarabine phosphate was not mutagenic to bacteria (Ames test) or mammalian cells (HGRPT assay in Chinese hamster ovary cells) either in the presence or absence of metabolic activation. Fludarabine phosphate was clastogenic *in vitro* to Chinese hamster ovary cells (chromosome aberrations in the presence of metabolic activation) and induced sister chromatid exchanges both with and without metabolic activation. In addition, fludarabine phosphate was clastogenic *in vivo* (mouse micronucleus assay) but was not mutagenic to germ cells (dominant lethal test in male mice).

### Impairment of Fertility

Studies in mice, rats and dogs have demonstrated dose-related adverse effects on the male reproductive system. Observations consisted of a decrease in mean testicular weights in mice and rats with a trend toward decreased testicular weights in dogs and degeneration and necrosis of spermatogenic epithelium of the testes in mice, rats and dogs. The possible adverse effects on fertility in humans have not been adequately evaluated.

### Pregnancy

*Pregnancy Category D:*

(see **WARNINGS** section).

### Nursing Mothers

It is not known whether fludarabine phosphate is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions including tumorigenicity in nursing infants, a decision should be made to discontinue nursing or discontinue the drug, taking into account the importance of the drug to the mother.

### Pediatric Use

Data submitted to the FDA was insufficient to establish efficacy in any childhood malignancy. Fludarabine was evaluated in 62 pediatric patients (median age 10, range 1-21) with refractory acute leukemia (45 patients) or solid tumors (17 patients). The fludarabine regimen tested for pediatric acute lymphocytic leukemia (ALL) patients was a loading bolus of 10.5 mg/m<sup>2</sup>/day followed by a continuous infusion of 30.5 mg/m<sup>2</sup>/day for 5 days. In 12 pediatric patients with solid tumors, dose-limiting myelosuppression was observed with a loading dose of 8 mg/m<sup>2</sup>/day followed by a continuous infusion of 23.5 mg/m<sup>2</sup>/day for 5 days. The maximum tolerated dose was a loading dose of 7 mg/m<sup>2</sup>/day followed by a continuous infusion of 20 mg/m<sup>2</sup>/day for 5 days. Treatment toxicity included bone marrow suppression. Platelet counts appeared to be more sensitive to the effects of fludarabine than hemoglobin and white blood cell counts. Other adverse events included fever, chills, asthenia, rash, nausea, vomiting, diarrhea, and infection. There were no reported occurrences of peripheral neuropathy or pulmonary hypersensitivity reaction.

### Vaccination

During and after treatment with FLUDARA FOR INJECTION, vaccination with live vaccines should be avoided.

### Disease Progression

Disease progression and transformation (e.g. Richter's syndrome) have been reported in CLL patients.

## ADVERSE REACTIONS

The most common adverse events include myelosuppression (neutropenia, thrombocytopenia and anemia), fever and chills, infection, and nausea and vomiting. Other commonly reported events include malaise, fatigue, anorexia, and weakness. Serious opportunistic infections have occurred in CLL patients treated with FLUDARA FOR INJECTION. Adverse events, and those reactions which are more clearly related to the

drug are arranged below according to body system.

#### Hematopoietic Systems

Hematologic events (neutropenia, thrombocytopenia, and/or anemia) were reported in the majority of CLL patients treated with FLUDARA FOR INJECTION. During FLUDARA FOR INJECTION treatment of 133 patients with CLL, the absolute neutrophil count decreased to less than 500/mm<sup>3</sup> in 59% of patients, hemoglobin decreased from pretreatment values by at least 2 grams percent in 60%, and platelet count decreased from pretreatment values by at least 50% in 55%. Myelosuppression may be severe, cumulative, and may affect multiple cell lines. Bone marrow fibrosis occurred in one CLL patient treated with FLUDARA FOR INJECTION.

Several instances of trilineage bone marrow hypoplasia or aplasia resulting in pancytopenia, sometimes resulting in death, have been reported in postmarketing surveillance. The duration of clinically significant cytopenia in the reported cases has ranged from approximately 2 months to approximately 1 year. These episodes have occurred both in previously treated or untreated patients.

Life-threatening and sometimes fatal autoimmune phenomena such as hemolytic anemia, autoimmune thrombocytopenia/thrombocytopenic purpura (ITP), Evan's syndrome, and acquired hemophilia have been reported to occur in patients receiving FLUDARA FOR INJECTION (see **WARNINGS** section). The majority of patients rechallenged with FLUDARA FOR INJECTION developed a recurrence in the hemolytic process.

In postmarketing experience, cases of myelodysplastic syndrome and acute myeloid leukemia, mainly associated with prior, concomitant or subsequent treatment with alkylating agents, topoisomerase inhibitors, or irradiation have been reported.

#### Infections

Serious, and sometimes fatal infections, including opportunistic infections and reactivations of latent viral infections such as VZV (Herpes zoster), Epstein-Barr virus and JC virus (progressive multifocal leukoencephalopathy) have been reported in patients treated with FLUDARA FOR INJECTION.

Rare cases of Epstein Barr Virus (EBV) associated lymphoproliferative disorders have been reported in patients treated with FLUDARA FOR INJECTION.

#### Metabolic

Tumor lysis syndrome has been reported in CLL patients treated with FLUDARA FOR INJECTION. This complication may include hyperuricemia, hyperphosphatemia, hypocalcemia, metabolic acidosis, hyperkalemia, hematuria, urate crystalluria, and renal failure. The onset of this syndrome may be heralded by flank pain and hematuria.

#### Nervous System

(See **WARNINGS** section) Objective weakness, agitation, confusion, seizures, visual disturbances, optic neuritis, optic neuropathy, blindness and coma have occurred in CLL patients treated with FLUDARA FOR INJECTION at the recommended dose. Peripheral neuropathy has been observed in patients treated with FLUDARA FOR INJECTION and one case of wrist-drop was reported.

In postmarketing experience, cases of progressive multifocal leukoencephalopathy have been reported. Most cases had a fatal outcome. Many of these cases were confounded by prior and/or concurrent chemotherapy. The time to onset has ranged from a few weeks to approximately one year after initiating treatment.

#### Pulmonary System

Pneumonia, a frequent manifestation of infection in CLL patients, occurred in 16%, and 22% of those treated with FLUDARA FOR INJECTION in the MDAH and SWOG studies, respectively. Pulmonary hypersensitivity reactions to FLUDARA FOR INJECTION characterized by dyspnea, cough and interstitial pulmonary infiltrate have been observed.

In postmarketing experience, cases of severe pulmonary toxicity have been observed with Fludara use which resulted in ARDS, respiratory distress, pulmonary hemorrhage, pulmonary fibrosis, and respiratory failure. After an infectious origin has been excluded, some patients experienced symptom improvement with corticosteroids.

#### Gastrointestinal System

Gastrointestinal disturbances such as nausea and vomiting, anorexia, diarrhea, stomatitis and gastrointestinal bleeding have been reported in patients treated with FLUDARA FOR INJECTION.

#### Cardiovascular

Edema has been frequently reported. One patient developed a pericardial effusion possibly related to treatment with FLUDARA FOR INJECTION. No other severe cardiovascular events were considered to be drug related.

#### Genitourinary System

Rare cases of hemorrhagic cystitis have been reported in patients treated with FLUDARA FOR INJECTION.

#### Skin

Skin toxicity, consisting primarily of skin rashes, has been reported in patients treated with FLUDARA FOR INJECTION.

Erythema multiforme, Stevens-Johnson syndrome, toxic epidermal necrolysis, and pemphigus have been reported, with fatal outcomes in some cases.

Worsening or flare up of pre-existing skin cancer lesions, as well as new onset of skin cancer, has been reported in patients during or after treatment with FLUDARA FOR INJECTION.

Data in the following table are derived from the 133 patients with CLL who received FLUDARA FOR INJECTION in the MDAH and SWOG studies.

**PERCENT OF CLL PATIENTS REPORTING  
NON-HEMATOLOGIC ADVERSE EVENTS**

<u>ADVERSE EVENTS</u>	<u>MDAH</u> (N=101)	<u>SWOG</u> (N=32)
ANY ADVERSE EVENT	88%	91%
BODY AS A WHOLE	72	84
FEVER	60	69
CHILLS	11	19
FATIGUE	10	38
INFECTION	33	44
PAIN	20	22
MALAISE	8	6
DIAPHORESIS	1	13
ALOPECIA	0	3
ANAPHYLAXIS	1	0
HEMORRHAGE	1	0
HYPERGLYCEMIA	1	6
DEHYDRATION	1	0
NEUROLOGICAL	21	69
WEAKNESS	9	65
PARESTHESIA	4	12
HEADACHE	3	0
VISUAL DISTURBANCE	3	15
HEARING LOSS	2	6
SLEEP DISORDER	1	3
DEPRESSION	1	0
CEREBELLAR SYNDROME	1	0
IMPAIRED MENTATION	1	0
PULMONARY	35	69
COUGH	10	44
PNEUMONIA	16	22
DYSPNEA	9	22
SINUSITIS	5	0
PHARYNGITIS	0	9
UPPER RESPIRATORY INFECTION	2	16
ALLERGIC PNEUMONITIS	0	6
EPISTAXIS	1	0
HEMOPTYSIS	1	6
BRONCHITIS	1	0
HYPOXIA	1	0
GASTROINTESTINAL	46	63
NAUSEA/VOMITING	36	31
DIARRHEA	15	13
ANOREXIA	7	34
STOMATITIS	9	0
GI BLEEDING	3	13
ESOPHAGITIS	3	0
MUCOSITIS	2	0
LIVER FAILURE	1	0

CHOLELITHIASIS	0	3
CONSTIPATION	1	3
DYSPHAGIA	1	0
CUTANEOUS	17	18
RASH	15	15
PRURITUS	1	3
SEBORRHEA	1	0
GENITOURINARY	12	22
DYSURIA	4	3
URINARY INFECTION	2	15
HEMATURIA	2	3
RENAL FAILURE	1	0
ABNORMAL RENAL FUNCTION TEST	1	0
PROTEINURIA	1	0
HESITANCY	0	3
CARDIOVASCULAR	12	38
EDEMA	8	19
ANGINA	0	6
CONGESTIVE HEART FAILURE	0	3
ARRHYTHMIA	0	3
SUPRAVENTRICULAR TACHYCARDIA	0	3
MYOCARDIAL INFARCTION	0	3
DEEP VENOUS THROMBOSIS	1	3
PHLEBITIS	1	3
TRANSIENT ISCHEMIC ATTACK	1	0
ANEURYSM	1	0
CEREBROVASCULAR ACCIDENT	0	3
MUSCULOSKELETAL	7	16
MYALGIA	4	16
OSTEOPOROSIS	2	0
ARTHRALGIA	1	0
TUMOR LYSIS SYNDROME	1	0

More than 3000 adult patients received FLUDARA FOR INJECTION in studies of other leukemias, lymphomas, and other solid tumors. The spectrum of adverse effects reported in these studies was consistent with the data presented above.

## OVERDOSAGE

High doses of FLUDARA FOR INJECTION (see **WARNINGS** section) have been associated with an irreversible central nervous system toxicity characterized by delayed blindness, coma and death. High doses are also associated with severe thrombocytopenia and neutropenia due to bone marrow suppression. There is no known specific antidote for FLUDARA FOR INJECTION overdose. Treatment consists of drug discontinuation and supportive therapy.

## DOSAGE AND ADMINISTRATION

### Usual Dose

The recommended adult dose of FLUDARA FOR INJECTION is 25 mg/m<sup>2</sup> administered intravenously over a period of approximately 30 minutes daily for five consecutive days. Each 5 day course of treatment should commence every 28 days. Dosage may be decreased or delayed based on evidence of hematologic or

non-hematologic toxicity. Physicians should consider delaying or discontinuing the drug if neurotoxicity occurs.

A number of clinical settings may predispose to increased toxicity from FLUDARA FOR INJECTION. These include advanced age, renal insufficiency, and bone marrow impairment. Such patients should be monitored closely for excessive toxicity and the dose modified accordingly.

The optimal duration of treatment has not been clearly established. It is recommended that three additional cycles of FLUDARA FOR INJECTION be administered following the achievement of a maximal response and then the drug should be discontinued.

#### Renal Insufficiency

Adult patients with moderate impairment of renal function (creatinine clearance 30-70 mL/min/1.73 m<sup>2</sup>) should have a 20% dose reduction of FLUDARA FOR INJECTION. FLUDARA FOR INJECTION should not be administered to patients with severely impaired renal function (creatinine clearance less than 30 mL/min/1.73 m<sup>2</sup>).

#### Preparation of Solutions

FLUDARA FOR INJECTION should be prepared for parenteral use by aseptically adding Sterile Water for Injection USP. When reconstituted with 2mL of Sterile Water for Injection, USP, the solid cake should fully dissolve in 15 seconds or less; each mL of the resulting solution will contain 25 mg of fludarabine phosphate, 25 mg of mannitol, and sodium hydroxide to adjust the pH to 7.7. The pH range for the final product is 7.2-8.2. In clinical studies, the product has been diluted in 100 cc or 125 cc of 5% Dextrose Injection USP or 0.9% Sodium Chloride USP.

Reconstituted FLUDARA FOR INJECTION contains no antimicrobial preservative and thus should be used within 8 hours of reconstitution. Care must be taken to assure the sterility of prepared solutions. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration.

FLUDARA FOR INJECTION should not be mixed with other drugs.

#### Handling and Disposal

Procedures for proper handling and disposal should be considered. Consideration should be given to handling and disposal according to guidelines issued for cytotoxic drugs. Several guidelines on this subject have been published.<sup>1-4</sup>

Caution should be exercised in the handling and preparation of FLUDARA FOR INJECTION solution. The use of latex gloves and safety glasses is recommended to avoid exposure in case of breakage of the vial or other accidental spillage. If the solution contacts the skin or mucous membranes, wash thoroughly with soap and water; rinse eyes thoroughly with plain water. Avoid exposure by inhalation or by direct contact of the skin or mucous membranes.

#### HOW SUPPLIED

FLUDARA FOR INJECTION is supplied as a white, lyophilized solid cake. Each vial contains 50 mg of fludarabine phosphate, 50 mg of mannitol, and sodium hydroxide to adjust pH to 7.7. The pH range for the final product is 7.2-8.2. Store under refrigeration, between 2°-8°C (36°-46°F).

FLUDARA FOR INJECTION is supplied in a clear glass single dose vial (6mL capacity) and packaged in a single dose vial carton in a shelf pack of five.

NDC 50419-511-06

Manufactured by: Ben Venue Laboratories, Bedford, OH 44146

Manufactured for:

Bayer HealthCare Pharmaceuticals Inc., Wayne, NJ 07470

U.S. Patent Number: 4,357,324

6700102

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<b>FLUDARA</b>			
fludarabine phosphate injection, powder, lyophilized, for solution			
<b>Product Information</b>			
Product Type	HUMAN PRESCRIPTION DRUG	NDC Product Code (Source)	50419-511
Route of Administration	INTRAVENOUS	DEA Schedule	
<b>INGREDIENTS</b>			
Name (Active Moiety)	Type	Strength	
fludarabine phosphate (fludarabine)	Active	50 MILLIGRAM In 2 MILLILITER	
mannitol	Inactive	50 MILLIGRAM In 2 MILLILITER	
sodium hydroxide	Inactive		
<b>Product Characteristics</b>			
Color		Score	
Shape		Size	
Flavor		Imprint Code	
Contains			
<b>Packaging</b>			
# NDC	Package Description	Multilevel Packaging	
1 50419-511-06	5 VIAL In 1 CARTON	contains a VIAL	
1	2 mL (MILLILITER) In 1 VIAL	This package is contained within the CARTON (50419-511-06)	

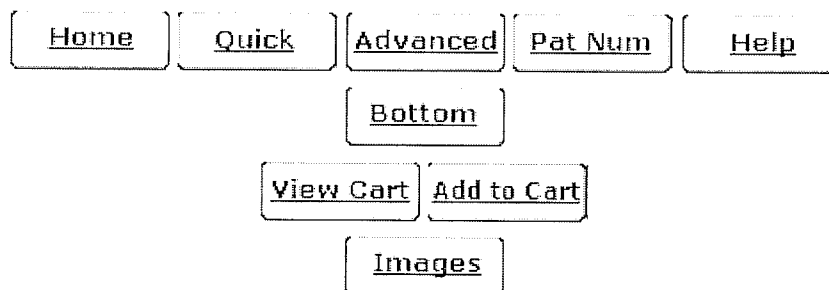
Revised: 03/2009

Bayer HealthCare Pharmaceuticals Inc.

# EXHIBIT H

## PATENT 4,808,576



USPTO PATENT FULL-TEXT AND IMAGE DATABASE

( 1 of 1 )

**United States Patent****4,808,576****Schultz , et al.****February 28, 1989**

Remote administration of hyaluronic acid to mammals

**Abstract**

The present disclosure is concerned with the discovery that hyaluronic acid, an agent well known to reduce the sequelae of trauma in mammalian joint tissue when applied directly to the traumatized tissue, will be carried to such traumatized tissue by the mammal's natural processes if applied at a site remote from the traumatized tissue. Thus, hyaluronic acid, in any therapeutically acceptable form, can be administered by the typical remote routes including intravenous, intramuscular, subcutaneous and topical. This makes the utilization of hyaluronic acid much more convenient and attractive. For instance the treatment of arthritis in horse or human joints with hyaluronic acid no longer requires more difficult intra articular injections.

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Assignee: **Mobay Corporation** (Pittsburgh, PA)

Appl. No.: **06/856,732**

Filed: **April 28, 1986**

**Current U.S. Class:****514/54 ; 514/825; 536/55.1****Current International Class:**

A61K 31/715 (20060101); A61K 9/08 (20060101); A61K 9/00 (20060101); C08B 37/00 (20060101); C08B 37/08 (20060101); A61K 031/715 ()

**Field of Search:****514/54,825 536/55.1****References Cited [Referenced By]****U.S. Patent Documents**4055660

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**Foreign Patent Documents**

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*Primary Examiner:* Griffin; Ronald W.

*Attorney, Agent or Firm:* Harsh; Gene Gil; Joseph C. Whalen; Lyndanne M.

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**Claims**


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What is claimed is:

1. A process for reducing the inflammation, pain or other result of trauma in irritated mammalian tissue comprising administering an effective dose of hyaluronic acid or a pharmacologically acceptable salt thereof at an effective site in or on the body of the mammal remote from said tissue.
2. The process of claim 1 wherein the dose is administered topically, subcutaneously, intramuscularly or intravenously.

3. The process of claim 2 wherein the irritated or inflamed tissue is present in a joint.
4. The process of claim 2 wherein the irritated or inflamed tissue is at the site of a surgical intrusion.
5. The process of claim 4 wherein the irritated or inflamed tissue is at the site of tendon surgery and the treatment reduces the incidence of adhesions.
6. The process of claim 2 wherein the inflammation is a result of an arthritic condition of a joint.
7. The process of claim 2 wherein the inflammation and soreness of a horse joint is at least partially alleviated.
8. The process of claim 7 wherein the dose is at least about 0.02 mg. per pound of body weight of the subject being treated.
9. The process of claim 7 wherein the dose is at least 0.04 mg per pound of body weight of the subject being treated.
10. The process of claim 2 wherein the dose is at least about 0.02 mg. per pound of body weight of the subject being treated.
11. The process of claim 9 wherein the dose is at least 0.08 mg per pound of body weight of the subject being treated.
12. The process of claim 10 wherein the hyaluronic acid is administered by an injection route as an aqueous 0.5 to 3.0 weight per cent solution having a 37.degree. C. viscosity of less than about 200 c/s.
13. The process of claim 10 or 12 wherein the hyaluronic acid has an FPLC determined molecular weight distribution lying almost entirely between 1.5 and 4 million daltons.
14. The process of claim 13 wherein the hyaluronic acid is non-pyrogenic and by UV absorbance has a combined amino acid and protein content of less than about 1.25 mg/ml and a nucleic acid content of less than about 0.06 mg/ml.
15. A process of at least partially alleviating inflammation or soreness or both of a horse joint comprising the intramuscular injection to such horse of an approximately one weight percent aqueous solution of sodium hyaluronate having a 37.degree. C. solution viscosity below about 200 c/s and a FPLC determined molecular weight distribution which is a single significant peak lying between about 1.5 and 4.0 million daltons at a dose of at least about 0.04 mg. of hyaluronate per pound of horse body weight.
16. The process of claim 15 wherein the treatment regimen comprises three injections of at least about 0.04 mg. of sodium hyaluronate each.
17. The process of claim 16 wherein the injections are given at four day intervals.
18. The process of claim 2 wherein the hyaluronic acid is administered topically in combination with a transdermal carrier.
19. The process of claim 18 wherein the 37.degree. C. viscosity of the treatment combination is in excess of about 1000 c/s.

20. The process of claim 18 wherein the transdermal carrier is selected from the group consisting of methyl salicylate, sodium salicylate, benzyl alcohol, oleic acid, 10% propylene glycol, 1% sodium glycolate, 1% polyoxyethylene-10-cetyether, 0.1% sodium EDTA, 1% sodium dodecyl sulfate and dimethyl sulfoxide.
21. The process of claim 20 wherein the transdermal carrier is dimethyl sulfoxide.
22. The process of claim 18 wherein the hyaluronic acid is between about 0.5 and 3.0 weight percent aqueous solution and is mixed with up to about 30 volume percent of a transdermal carrier.
23. The process of claim 22 wherein the hyaluronic acid has a FPLC determined molecular weight of at least 5.times.10.sup.5 daltons, a combined amino acid and protein content of less than about 1.25 mg/ml by UV absorption and a nucleic acid content of less than about 0.06 mg/ml by UV absorption.
24. The process of claim 23 wherein the hyaluronic acid has a FPLC determined molecular weight distribution 98% of which lies in a single symmetrical peak between about 1.5 and 4.0.times.10.sup.6 daltons.
25. The process of claim 24 wherein the hyaluronic acid is in the form of its sodium hyaluronate salt.
26. The process of claim 18 wherein the dose is in excess of about 0.10 milligrams per pound of body weight of the mammal being treated.
27. A process of relieving joint or muscle pain in a mammal comprising the topical application to such mammal, at an effective site of a mixture of between about 0.5 and 2.5 weight percent of a non-pyrogenic aqueous sodium hyaluronate solution having a UV absorbance determined combined amino acid and protein content of less than 1.25 mg/ml, a UV absorbance determined nucleic acid content of less than about 0.06 mg/ml, and a FPLC determined average molecular weight of greater than about 5.times.10.sup.5 daltons and up to about 30 volume percent, based on the volume of the mixture, of a transdermal carrier at a dosage of greater than about 0.10 mg per pound of body weight of the mammal being treated.
28. The process of claim 27 wherein the transdermal carrier is selected from the group consisting of methyl salicylate, sodium salicylate, benzyl alcohol, oleic acid, 10% propylene glycol, 1% sodium glycolate, 1% polyoxyethylene-10-cetyether, 0.1% sodium EDTA, 1% sodium dodecyl sulfate and dimethyl sulfoxide.
29. The process of claim 27 wherein the sodium hyaluronate has a UV absorbance determined combined amine acid and protein content of less than about 0.1 mg/ml, a UV absorbance determined nucleic acid content of less than about 0.005 mg/ml and a FPLC determined molecular weight distribution 98% of which lies in a single symmetrical peak between about 1.5 and 4.0.times.10.sup.6 daltons.
30. The process of claims 27, 28 or 29 wherein the mammal is man.
31. The process of claims 27, 28 or 29 wherein the mammal is selected from the group consisting of the horse, the dog and the cat.
32. The process of claim 27 wherein the application is made in the vicinity of the origin of the pain.
33. The process of claim 27 wherein the application is made at a site substantially removed from the

source of the pain.

34. A process for the at least partial alleviation of arthritic symptoms in a mammalian joint comprising the administration to such mammal of an effective dose of hyaluronic acid or a pharmacologically acceptable salt thereof at an effective site in or on the body of the mammal remote from the arthritic joint.

35. A process for reducing the tendency of adhesions to form at the site of a surgical intrusion into a mammalian body comprising the administration to such mammal of an effective dose of hyaluronic acid or a pharmacologically acceptable salt thereof at an effective site on or in the body of the mammal remote from the surgical intrusion.

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### *Description*

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#### FIELD OF THE INVENTION

The present invention is concerned with the discovery that a known therapeutic agent, hyaluronic acid, can be effectively administered to mammals by techniques which require the mammal's internal bodily processes to transport this high molecular weight agent to the site of action.

#### BACKGROUND OF THE INVENTION

Hyaluronic acid is a well known mucopolysaccharide which is found in the joint tissue and vitreous humor of the eyes of mammals. It has been extracted from rooster combs and human umbilical cords and bacterial cultures such as those of hemolytic group A and C streptococci for various therapeutic purposes. The initial therapeutic use of this material was as a replacement for the liquid vitreous of the human eye to aid in ophthalmic surgery, especially in the treatment of retinal detachment. It has also found utility for the relief of trauma or irritation in joint tissue of mammals including humans by injection into the synovial fluid of the joint. It has been proposed that it be used both as a primary medicant and as an auxiliary with other joint medicines. An extensive discussion of its various utilities is found in U.S. Pat. No. 4,141,973 to Balazs. The use of hyaluronic acid alone and with cortisone in various animal joints, especially horses, is discussed in the "Effect of Intra-articular Injection of Hyaluronic Acid on the Clinical Symptoms of Osteoarthritis and on Granulation Tissue Formation" by Rydell et al. and appearing at pages 25 to 32 of the October, 1971 (Number 80) issue of Clinical Orthopaedics and Related Research. The use of hyaluronic acid in human joints is reported in the "Preliminary Clinical Assessment of Na Hyaluronate Injection into Human Arthritic Joints" by Peyron et al and appearing at pages 731 to 736 of the October 1974 (Volume 22, Number 8) of Pathologie Biologie. Finally, the use of hyaluronic acid in reducing fibrotic wound reactions is reported in the "Decreased Granulation Tissue Reaction After Installment of Hyaluronic Acid" by Rydell and appearing at pages 307 to 311 of Volume 41 of Acta Orthop. Scandinav.

The intra-articular use of hyaluronic acid in horse joints has been commercially promoted in connection with Pharmacia's Hylartil and Hylartin V product and Sterivet's Synacid product. However, the commercial attractiveness has been limited by the need to administer these products by injection into the affected joint.

A related material, a polysulfated glycosaminoglycan, has recently been introduced to the U.S. market by Luitpold Pharmaceuticals under the tradename Adequan.RTM. (also known in Europe as Arteparon) for the treatment of arthritic joints in horses. Initially the recommended route of administration was



intra-articular with a 250 mg injection each week for five weeks. A letter appearing at pages 446 and 447 of the April 1984 issue of Veterinary Medicine suggests that this material can be administered intramuscularly at approximately double the dosage on a four day interval for four to five weeks.

Polysulfated glycoaminoglycans have also been reported to stimulate the biosynthesis of hyaluronic acid in the synovial membranes of rabbit knee joints thus suggesting that although the mode of action may be different from hyaluronic acid the same conditions might be beneficially effected. Interestingly the report, "Influences of Sulfated Glycosaminoglycans on Biosynthesis of Hyaluronic Acid in Rabbit Knee Synovial Membrane", by Nishikawa et al and appearing at pages 146 to 153 of the July, 1985 issue (Volume 240) of Arch. Biochem. Biophys. indicates that hyaluronic acid itself has no such stimulatory effect.

This stimulatory agent has been reported to be effective if administered either intramuscularly to humans or subcutaneously to rats. The former effect is noted in "Vergleich von Glykosaminoglykanpolysulfat (Arteparon) und physiologischen Kochsalzloesung bei Arthrosen grosser Gelenke. Ergebnisse einer multizentrischen Doppelblindstudie" by Siegmeth et al and appearing at pages 223 to 228 of the July/August 1983 issue (Volume 42, Number 4) of Z. Rheumatol. The latter effect is discussed in "Die Tierexperimentelle Gonarthrose Der Ratte und Ihre Therapie mit Glyko-aminoglykan polysulfat" by Buchmann et al and appearing at pages 100 to 107 of the 1985 issue (Volume 44, Number 3) of Z. Rheumatol.

This stimulatory agent has also been reported to have a broad range of lower molecular weights. In the "Influence of a Glycosaminoglycan Polysulfate (Arteparon) on Lysosomal Enzyme Release from Human Polymorphonuclear Leukocytes" by Mikulikova and appearing at pages 50 to 53 of the March/April 1982 issue (Volume 41, Number 2) of Z. Rheumatol is an indication that Arteparon can be fractionated into portions having molecular weights between 3000 and 17,000. In "Polysulfated Glycosaminoglycan: a New Intra-articular Treatment for Equine Lameness" by Hamm, Goldman and Jones appearing at pages 811 to 816 of the June, 1984 issue of Veterinary Medicine Adequan is reported to have an approximate molecular weight of 10,000. In this regard, one of the coauthors, Dr. Goldman, is associated with Lutipold Pharmaceuticals, the U.S. distributor of this product.

The direct use of hyaluronic acid has been found to be effective at lower dosages than this polysulfated glycosaminoglycan, which stimulates hyaluronic acid production in joint tissue. For instance the Adequan polysulfated glycosaminoglycan is sold with a dosage recommendation of 250 mg for intra-articular administration to horses whereas Pharmacia markets hyaluronic acid under the name Hylartil with a recommended dosage for intraarticular administration of 20 mg. Although the Adequan product has been reported to be effective when administered intramuscularly at double the dose (500 mg) a similar effect was not expected for the high molecular weight sodium hyaluronate.

It was not expected that sodium hyaluronate would be amenable to remote administration because its primary action was thought to be lubrication and because of its molecular weight which is typically well in excess of 1.times.10.sup.6 daltons. It was expected that molecules of such a molecular weight would be too large to transfer through mammalian tissue to the remote site of trauma. Thus, it was assumed that effective amounts of sodium hyaluronate could not be transported through the body of a mammal to the site of action upon the remote administration of reasonable sized doses.

It has now been found that the remote administration of hyaluronic acid is effective in reducing the pain and swelling of traumatized or irritated mammalian tissue, particularly joint tissue.

## SUMMARY OF THE INVENTION

A process for reducing the sequela of the trauma in irritated or inflamed mammalian tissue by the remote administration of hyaluronic acid or a pharmacologically acceptable salt thereof has been discovered. (Hereinafter for convenience the term hyaluronic acid is used to denote both the free acid and the pharmacologically acceptable salts thereof interchangeably except where otherwise explicitly indicated). The hyaluronic acid is introduced to the body of the mammal at other than the site of the traumatized tissue and is effectively transported to the site of action by the body's internal processes. This allows the use of such convenient routes of administration as intramuscular, intravenous, subcutaneous, and topical. Two particularly preferred routes of administration are intramuscular injection and topical application in a recognized transdermal carrier and a particularly amenable condition for such treatment is irritated or inflamed joint tissue.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a set of plots of the change in carpal joint circumference versus the time after treatment with hyaluronic acid for treated and control horses with Freund's Complete Adjuvant induced trauma in the carpal joint.

FIG. 2 is a set of plots of the change in range of motion versus the time after treatment for the same horses as in FIG. 1.

FIG. 3 is a set of plots of the change in range on motion normalized to the day of treatment versus the time after treatment for the same horses as in FIG. 1.

FIG. 4 is the same set of plots as in FIG. 3 but with all ordinate values arbitrarily increased by 23.7% to set the lowest point at 0%.

FIG. 5 is a set of plots of lameness index versus time after treatment for the same horses as in FIG. 1.

FIG. 6 is a set of plots of the per cent improvement in stride versus time after treatment for the same horses as in FIG. 1.

#### DETAILED DESCRIPTION OF THE INVENTION

The trauma in irritated or inflamed mammalian tissue is reduced by applying hyaluronic acid by any of the accepted routes of administration except direct application to the affected tissue. A particularly interesting embodiment involves the treatment of joint tissue. The direct application of hyaluronic acid involves intra-articular injection which is a procedure requiring considerable care and skill in the larger joints of larger mammals such as the leg joints of horses. The treatment of smaller mammals such as dogs and cats and the smaller joints of larger mammals such as human finger joints requires correspondingly greater care and skill. Treatment by remote administration such as intramuscular, intravenous or subcutaneous injection or topical application in a transdermal carrier in such cases is much more convenient and attractive. However, the fact that the internal transport systems of the mammalian body are effective in conveying hyaluronic acid to the affected site makes it possible to treat other traumatized tissue by remote application as well. Thus, remote administration can be used to treat the other conditions for which hyaluronic acid has found utility such as the post surgical adhesions associated with incisions and tendon repair reported on in the Rydell et al article appearing at pages 25 to 32 of the October 1971 issue of Clinical Orthopedics and Related Research.

The hyaluronic acid useful in the treatment of irritated or inflamed tissue by remote application may be of any type already recognized as useful for such purposes. It may be extracted from animal tissue such as rooster combs or umbilical cords or from bacterial cultures such as those of hemolytic group A or C

streptococci. It should be pure enough to avoid provoking an adverse or toxic reaction in the mammal being treated. This implies that it is free of pyrogens and has a sufficiently low level of proteins and nucleic acids that no substantial immune reaction is provoked. It is preferably of high molecular weight and is also preferably of low viscosity for the injection routes. The polymer may be in its free acid form or in any pharmacologically acceptable salt form.

The preferred source of hyaluronic acid is a culture of an appropriate microorganism. The use of the culturing and harvesting techniques described in European Published Patent Application No. 144,019 are particularly valuable in obtaining material with desirable purity and molecular weight. Among the organisms to which these techniques can be applied the Group A and Group C streptococci are preferred with the Group C being especially preferred and the *Streptococcus equi* being most preferred. Further preferred is the hyaluronic acid obtained according to the teachings of copending U.S. patent application Ser. No. 816,548 filed Jan. 6, 1986.

Both the protein and amino acid content, and the nucleic acid content of the hyaluronic acid should be carefully controlled because both are known to display antigenic activity in mammals. The content of both are conveniently monitored and evaluated by UV absorbance with the former correlated to the optical density at 280 nanometers and the latter to the optical density at 257 nanometers. It is preferred that the content of the former be less than about 1.25, especially less than about 0.1, mg/ml and that the content of the latter be less than about 0.06, especially less than about 0.005, mg/ml. In this regard the absorbance at 280 nanometers does not distinguish between amino acids and proteins. However, while amino acids alone are non-antigenic, they readily complex with hyaluronic acid and the complex may readily provoke an immune response in mammals. Therefore in the context of the present technology it is desirable to control the content of both and thus it is appropriate to specify a maximum content for the combination of both which is correlated with a particular UV absorbance. In an especially preferred hyaluronic acid the total amino acid content as measured by the orthophthalaldehyde fluorescence technique (which inherently involves the hydrolysis of any protein present back to its constituent amino acids) is less than about 0.4 mg/ml and the nucleic acid content is less than about 0.06 mg/ml as measured by the ethidium bromide fluorescence technique.

The hyaluronic acid can be utilized in its free acid form or in any pharmacologically acceptable salt form. One of the most convenient forms is as the sodium salt because this polymer is typically purified by successive precipitations in ethanol or other organic solvents and dissolutions in water and the sodium salt is particularly amenable to such procedures. In fact, all the limitations on purity, viscosity and molecular weight discussed herein were developed on the sodium salt as were the specific application data discussed hereinafter. However, the remote application development is equally applicable to other forms such as the free acid or potassium salt. For convenience the discussion herein includes all these forms within the term hyaluronic acid.

The hyaluronic acid should have a high average molecular weight. Although forms of this material with average molecular weights of 55,000 or less are known the preferred hyaluronic acid has an average molecular weight of at least 5.times.10<sup>5</sup> determined by FPLC (fast protein liquid chromatography) in accordance with the technique disclosed in copending U.S. patent application Ser. No. 816,548 filed Jan. 6, 1986. Average molecular weights in excess of about 1.0.times.10<sup>6</sup> preferably 1.2.times.10<sup>6</sup>, and especially in excess of about 1.8.times.10<sup>6</sup> are particularly preferred. It is further preferred that the hyaluronic acid display a fairly narrow molecular weight distribution and a distribution with a single gel permeation peak is particularly preferred. A single symmetrical FPLC peak with 98% of the molecules having a weight between about 1.2.times.10<sup>6</sup> and 4.0.times.10<sup>6</sup> is especially preferred.

The hyaluronic acid may have either a high or a low viscosity depending on what is convenient for the



route of treatment desired. The higher viscosities are convenient for topical applications while the lower viscosities are convenient for the injection routes of administration, i.e. intra-muscular, intravenous or subcutaneous. The higher molecular weight hyaluronic acid may advantageously have viscosities between about 900 and 5000 centistokes per second (c/s) at 37.degree. C. for topical applications and advantageously have viscosities less than about 500, preferably less than 150 c/s at 37.degree. C. for other routes of administration. In both cases the viscosity is conveniently measured as a 1 wt. % aqueous solution of the sodium salt in a Cannon-Manning Semi-Micro Viscometer according to the procedures in ASTM D 445 and D 2515. The low viscosity material greatly facilitates the injection routes of administration by allowing for instance the use of reasonably concentrated aqueous sodium hyaluronate solution in practical size doses. Thus, a 1% aqueous solution of sodium hyaluronate can be readily utilized for injection doses of about 10 milliliters which contain about 100 milligrams of active ingredient if its viscosity is less than about 200 c/s at 37.degree. C.

The treatment of irritated or inflamed mammalian tissue by remote administration requires a dose or total dose regimen effective to reduce or alleviate the trauma. It is preferred to administer at least about 0.02 milligrams of hyaluronic acid per pound of body weight of the mammal being treated which is equivalent to about 0.044 milligrams per kilogram. It is particularly preferred to utilize at least about 0.04 and especially 0.08 milligrams per pound of body weight. In the case of topical application it is particularly desirable to use in excess of about 0.10, especially about 0.15, milligrams per pound of body weight. In as much as hyaluronic acid is a naturally occurring substance in mammals it is believed that there is no inherent upper limit to the tolerable dose. However, as in all medicinal treatments, it is prudent to use no more than is necessary to achieve the desired effect. Furthermore, any impurities which are at a low enough level to be well tolerated at effective dosages may provoke adverse reactions at unwarrantedly high dosages.

The topical treatment should be made by application of the hyaluronic acid combined with a compatible transdermal carrier. Any recognized carrier such as methyl salicylate, sodium salicylate, benzyl alcohol, oleic acid, 10% propylene glycol, 1% sodium glycolate, 1% polyoxyethylene-10-cetyl ether, 0.1% sodium EDTA, 1% sodium dodecyl sulfate, or dimethyl sulfoxide (DMSO) is suitable with DMSO being particularly preferred. A convenient application formulation is a mixture of a less than about 3 weight percent aqueous solution of hyaluronic acid, particularly as sodium hyaluronate, with an effective amount of transdermal carrier. A preferred formulation involves an aqueous between about 0.5 and 2.5 weight percent hyaluronate solution and up to about 30 volume percent of transdermal carrier. The hyaluronate solution and the total formulation both conveniently display a viscosity in excess of about 1000 c/s at 37.degree. C.

The present treatment has been found to be particularly effective in the treatment of joint distress in large mammals including that caused by arthritic conditions. Especially preferred applications involve the treatment of horses and man by intramuscular injection and topical administration in a transdermal carrier. A particularly effective treatment for joint pain in the leg joints, particularly the carpal and tibiotarsal (or hock) joints, of horses is an intramuscular injection preferably in the neck muscle. A particularly effective treatment for musculoskeletal pain in man is topical application in a transdermal carrier such as dimethyl sulfoxide (DMSO). The application may be in the vicinity of the affected joint tissue or it may be considerably more remote.

The remote application techniques of the present invention can also be utilized to alleviate any other condition against which hyaluronic acid is recognized as effective. Among these are the reduction or prevention of adhesions at the site of surgical intrusion, especially in the case of surgery involving tendons.

The remote application techniques of the present invention are of particular interest with regard to those

mammals which are among the commonly recognized companions to man. The amelioration of pain or discomfort in these companion animals is of the greatest interest and is the most practical among all treatable mammals. Of particular interest in this group are the cat, dog and horse.

The invention is further illustrated but is not intended to be limited by the following examples.

#### EXAMPLE 1

An investigation of the intramuscular treatment of horses.

A study involving eight mixed breed female and gelding horses was undertaken to determine if the intramuscular injection of sodium hyaluronate could alleviate the symptoms induced by the intra-articular injection of Freund's Complete Adjuvant into the intercarpal joint. This is a common and well accepted model for the study of joint distress, particularly arthritic conditions, in horses. The study established that three intramuscular injections of 0.08 milligrams per pound of horse weight given in the neck muscle 5, 9 and 13 days after inducement of joint distress with a 0.7 milliliter injection of the Adjuvant was effective.

The eight horses were initially acclimated to the housing facilities and testing apparatus for several days and then evaluated against the test criteria of joint circumference, range of motion, stride and lameness to provide a base score. The following day all eight horses were given a 0.7 milliliter injection of Freund's Complete Adjuvant in the left intercarpal joint. The horses displayed soreness in the left front leg over the next four days. On the fourth day after the intra-articular injection of the irritant the horses were re-rated against the same four criteria. A total score was developed for each horse consisting of the sum of the differences in each parameter except stride between the two measurements. These scores were then used to assign four horses to a control group and four to a treatment group by assigning the horse with the highest score to one group, the horses with the next two highest scores to the other group, the horses with the next two highest scores to the first group, the two horses with the next score to the second group and the last horse to the first group. The first group was designated the control group by an arbitrary toss of a coin. The table of measurements was as follows with measurement days arbitrarily designated -6 and -1:

														Horse			
Circumference	Range of Motion	Lameness	Stride	No.	-6	-1	Diff.	-6	-1	Diff.	-6	-1	Diff.	Total	-6	-1	Diff.
Total																	1
31.9	36.2	4.3	155.degree.	65.degree.	90.degree.	0	4	4	98.3	157.1	138.5	18.2	116.5	2	29.4	34.4	5.0
150.degree.	55.degree.	95.degree.	0	5	5	105.0	161.5	76.1	85.4	190.4	3	29.3	33.5	3.7	160.degree.		
30.degree.	130.degree.	0	5	5	138.7	151.1	78.5	82.8	221.5	4	30.0	35.5	5.5	195.degree.	45.degree.		
100.degree.	0	5	5	110.5	160.4	0	160.4	270.9	5	29.3	33.4	3.6	150.degree.	45.degree.	105.degree.	0	4
112.6	163.1	126.7	36.4	149.0	6	31.5	36.2	4.7	190.degree.	35.degree.	105.degree.	0	4	4	113.7	161.3	145.7
15.6	134.2	7	30.5	36.0	5.5	150.degree.	55.degree.	95.degree.	0	5	5	105.5	145.4	100.9	44.5	150.0	8
34.5	3.5	150.degree.	60.degree.	90.degree.	0	5	5	98.5	157.9	114.7	40.2	138.7					
														Ranking			
Horse No.	Group I	Control	Group II	Treatment													
																	1
138.7	6	113.7	2	6	113.7	4	110.5	5	112.6	3	5	112.6	7	105.5	2	105.0	4
Mean Score	113.25	Mean Score	107.45	6	2	105.0	7	8	98.5	8	1	98.3					3

On the next day (day 0) each of the horses was given an injection in the neck muscle of between 7.5 and 9.5 milliliters, depending on body weight. Each horse received an injection to the nearest 0.5 ml of 0.008

milliliters per pound of weight. The injection for the treatment horses consisted of a sterile 1.19 wt. % aqueous solution of sodium hyaluronate having a FPLC determined average molecular weight of  $1.88 \times 10^6$ , a nucleic acid content of less than 0.003 mg/ml by ethidium bromide fluorescence, a total amino acid content by orthophthalaldehyde fluorescence of less than 0.005 mg/ml and a 37 degree C. viscosity of 147 c/s while the injection for the control horses consisted of a sterile phosphate buffered saline solution. The weight of each horse and the dosage given to it at this time and four and eight days subsequently was as follows:

HORSE WEIGHT IN LBS		DOSE IN ML	
1190	9.5	21015	8.0
3985	8.0	41215	9.5
51030	8.0	6	

The injection site was palpated and the body temperature was taken daily for three days after the first injection and at the time of the second and third injections and finally four days after the third injection. The IM treatment produced no deleterious effect. All temperatures remained normal and there was no clinically significant injection site reaction (the Freund's Complete Adjuvant is a "known pyrogen" so temperature normality was judged by the fact that the treatment injections did not cause any further temperature elevation and did not appear to interfere with the drop in temperature from the peak induced by the traumatizing injection).

Each horse was evaluated against the criteria of joint circumference, range of motion, stride length and observed lameness seven, fourteen, twenty-one, twenty-eight, thirty-five and forty-two days after the first injection. These were the same parameters as had been used to classify the horses into the treatment and control groups and were defined as follows:

### Joint Evaluation Procedure

### 1. Joint Circumference

Joint circumference was measured while at the stall before exercise. It was measured at a point directly over the accessory carpal bone. It was measured with a cloth tape and recorded in centimeters.

## 2. Range of Motion

Range of Motion was measured at the stall. It was the difference between the angle of the affected leg at rest and the flexed angle. All three values were recorded. A goniometer was used to determine the angles.

- Leg at rest: With the horse in a standing position, the goniometer measured the angle of the carpus.
- Flexed angle: The affected joint was flexed while the leg was raised from the ground. The goniometer measured the angle at the point where the horse reacted to the flexion by flinching, shying or pulling back.

### 3. Stride Length

Stride length was measured before being placed on the walker. A long paper roll (20 feet) was used to record the distance between toe marks. Prior to walking across the paper, the toe of the affected leg was slosed with water or mineral oil. Two steps were recorded and the distance between the toe mark from the affected leg was measured in centimeters. The horse was walked across the paper three times. An average of the three measurements was used for the final value.

#### 4. Observed Lameness

The horse was placed on the mechanical walker and walked at 6 m.p.h. for five minutes. The score is determined while on the walker. The direction of the mechanical walker was such that the affected leg was to the inside.

0=No Lameness

1=Difficult to observe; not consistently apparent regardless of circumstances (i.e., weight-carrying, circling, inclines, hard surface, etc.).

2=Difficult to observe at a walk or trotting a straight line; consistently apparent under certain circumstances (i.e., weight-carrying circling, inclines, hard surface, etc.).

3=Consistently observable at a trot under all circumstances.

4=Obvious lameness; marked nodding, hitching or shortened stride.

5=Minimal weight-bearing in motion and/or at rest: inability to move.

If a horse was very reluctant to move, and placement on the mechanical walker was not advised, a score of 5 was recorded and the horse was returned to the stall.

An analysis of these parameters clearly demonstrated the beneficial result of the intramuscular injections of the hyaluronic acid in relieving the trauma induced by the Freund's Complete Adjuvant. The treatment horses displayed clearly superior performance to the control horses in every parameter but joint circumference for which the results were not as conclusive. This is in accord with other studies on the relief of trauma in horse joints wherein joint circumference was found not to be a particularly sensitive measurement parameter. The results are tabulated in the following table in which the "day" is the days before or after the first injection:

Horse Day																		
No.	Parameter	-6	-1	7	14	21	28	35	42	Treatment								
8	Circum.	31.0	34.5	36.4	36.7	36.1	35.6	35.2	35.8	2 (in. cm)	29.4	34.5	33.5	34.5	34.6	33.8	34.2	34.0
29.8		33.4	37.2	36.3	37.7	35.5	36.2	35.5	6	31.5	36.2	36.6	35.5	35.8	34.0	35.3	34.5	Control 3
35.7		36.8	36.8	37.2	36.3	39.3	4	30.0	35.5	35.5	36.4	36.3	35.3	36.5	34.0	1	31.9	36.2
39.4		38.8	7	30.5	36.0	36.6	36.8	37.7	36.4	39.2	40.0	Treatment 8	Range of 150.degree.	60.degree.				
40.degree.		70.degree.	95.degree.	100.degree.	100.degree.	95.degree.	2	Motion	150.degree.	55.degree.								
75.degree.		95.degree.	95.degree.	110.degree.	95.degree.	100.degree.	5	150.degree.	45.degree.	65.degree.								
70.degree.		70.degree.	70.degree.	75.degree.	70.degree.	6	140.degree.	35.degree.	85.degree.	115.degree.								
130.degree.		110.degree.	115.degree.	105.degree.	Control 3	160.degree.	30.degree.	50.degree.	45.degree.									
40.degree.		55.degree.	75.degree.	55.degree.	4	145.degree.	45.degree.	50.degree.	55.degree.	60.degree.								
80.degree.		75.degree.	85.degree.	1	155.degree.	65.degree.	85.degree.	75.degree.	95.degree.	90.degree.								
75.degree.		75.degree.	7	150.degree.	55.degree.	85.degree.	85.degree.	55.degree.	75.degree.	75.degree.								
55.degree.		Treatment 8	Lameness	0	5	4	1	0	1	0	0	2	0	5	3	1	1	1
Control 3		0	5	4	4	4	4	4	0	5	5	4	4	3	1	1	1	0
114.7		136.7	148.2	144.9	149.0	149.5	154.0	2 (in inches)	161.5	76.1	148.8	155.0	145.3	157.4	156.8	162.6		
5		163.1	126.7	135.8	156.9	153.4	155.4	159.8	169.3	6	161.3	145.7	149.2	161.8	168.2	164.7	165.2	167.1
Control 3		157.3	74.5	106.5	123.6	128.5	130.0	111.2	128.2	4	160.4	0*	108.9	142.9	143.7	152.1	147.1	



151.7 1 157.7 139.5 145.2 163.8 160.7 152.0 164.9 165.0 7 145.4 100.9 142.5 133.3 144.9 139.3 159.0  
145.9

\*A

zero indicates that the horse did not put the affected foot to the paper thus no stride could be measured.

Analyses of these results were plotted for each parameter along with some data on the intra-articular treatment of horse joints similarly traumatized by the injection of Freund's Complete Adjuvant. However, the induced trauma was somewhat less severe than that of the present study so the control horses from this study had a range of motion not too different than the treatment horses of the present study. But, more importantly, the difference in recovery of range of motion and lameness between treatment and control horses for the present study closely paralleled that observed in the earlier study. Thus, the effects obtained by intramuscular injection were highly similar to those previously obtained by intra-articular administration.

These analyses are displayed in FIGS. 1 through 6. In these figures the open squares represent the average values for the four treatment horses and the open triangles represent the average values for the four control horses of this study. The hexagons and shaded squares represent the average values for horses with a similarly induced joint trauma who were treated intra-articularly with a single dose of 40 and 20 milligrams, respectively, of a similar aqueous sodium hyaluronate solution while the shaded triangles represent the average values for the untreated control horses used in this earlier study.

FIG. 1 displays the change in the "joint circumference" from the day before the first injection until the end of the study forty three days later (for convenience the "day -1" value has been plotted on the 0 axis and represents the increase in joint circumference (in inches) since the day before the traumatizing injection, i.e. day -6). Although the joint swelling is never completely reversed, it is stabilized at a lower level or partially alleviated by hyaluronic acid treatment. The effect is somewhat less dramatic with the present (intramuscular) route of administration but is clearly present and is on joints which were indicated by the other parameters to be more severely traumatized than those treated intra-articularly.

FIG. 2 displays the change in the "range of motion" (ROM) over the same time period as FIG. 1. The 100% value is based on the ROM displayed the day before traumatization, i.e. on day -6. It is clear that the joints evaluated in the comparison intra-articular study were less traumatized because the inhibition of the ROM before treatment was less severe.

It is also clear that the intramuscular and the intra-articular treatments have caused parallel improvements over their respective controls. However, because the Freund's Complete Adjuvant causes an extreme trauma neither the inventive or the comparison treatment could completely alleviate the induced condition.

FIGS. 3 and 4 display further analyses of the ROM data wherein the percent of improvement over the condition the day before the first treatment injection is evaluated. The time scale is the same as in FIGS. 1 and 2. In FIG. 4 the graph has been arbitrarily adjusted upward by adding 23.7% to all ordinate values. Here it appears that the present invention's intramuscular administration results in a greater improvement over its control than in the case of the intra-articular administration.

FIG. 5 displays the change of the "observed lameness" over the same period as the previous figures. The intramuscular and the intra-articular routes of administration displayed approximately equivalent effects. The controls for the present study showed a somewhat higher degree of lameness suggesting that the traumatization for the present study was somewhat more severe than for the prior intra-articular study. The fact that the same level of lameness was obtained from both routes of administration suggests the route of the present invention might be somewhat more effective.

FIG. 6 displays the change in "stride length" over the same time period as the previous Figures. The stride at each evaluation was compared to the average value displayed the day before the traumatizing injection, i.e. day -6, to obtain a percentage of recovery. The horses treated according to both the present invention and intra-articular injection show a faster and greater ultimate recovery of stride compared to their respective controls.

## EXAMPLE 2

Back pain in a man aged 48, weight 155 pounds was alleviated by topical application of an aqueous solution of sodium hyaluronate mixed with 10 to 30 volume per cent of dimethyl sulfoxide (DMSO). In particular preparations were made up with 10, 20 and 30 volume per cent of DMSO and the balance a 1.7 weight per cent solution of sodium hyaluronate with a 37.degree. C. viscosity of in excess of 500 c/s and a FPLC determined molecular weight of 2.times.10.sup.6 daltons with a nucleic acid content of 0.00137 mg/ml and a total amino acid content by orthophthalaldehyde fluorescence of 0.0047 mg/ml. Applications of about 2 ml of preparation were made to the back in the vicinity of the pain twice daily for a period of two weeks. In each case the pain was relieved in about 15 minutes and the relief lasted about 8 to 10 hours. Treatment was then suspended and the pain returned in about four to five days. At this time treatment was resumed by the daily application of about 2 ml of the preparation to the knuckles of the hands with effective relief of the pain. This treatment was discontinued after three days and the pain did not reoccur in the next two and a half weeks. Thus, the pain was effectively treated with a topical dose of about 0.15 milligrams per pound of body weight (2 ml.times.1.7 wt. %.times.70 vol. %.times.1000 mg/ml) applied either in the vicinity of the pain or at a more remote location.

The pain relieved by this treatment had been close to constant for about two years before treatment was initiated. After treatment the pain was observed at a much lower intensity only after sitting or sleeping in a constant position for a prolonged period.

The topical application of the sodium hyaluronate without a transdermal carrier was ineffective. The hyaluronate solution simply evaporated to dryness leaving a film on the skin of the subject.

The application of equivalent amounts of DMSO alone was ineffective to relieve the back pain. In fact, it caused some irritation of the skin area to which it was applied. This was in distinct contrast to the application in combination with the sodium hyaluronate solution in which no skin irritation was observed.

## EXAMPLE 3

Various joint and muscle pains were relieved in a man age 54 weighing 250 pounds by the topical application of the same aqueous sodium hyaluronate used in Example 2 combined with 20 volume per cent of DMSO, based on total volume. A knee causing pain from several year old cartilage damage was treated with 4 milliliters of this preparation and relief was observed within 30 minutes. Treatment was repeated on four day intervals to successfully control the pain for about two weeks. Bursitis pain in a shoulder was successfully alleviated within a few minutes on the topical application of about 5 ml of this same preparation. Pain returned in about four days and was relieved by the same treatment. The cycle was repeated four times. Pain from sore muscles in the clavicle region was relieved by the application of 3 milliliters of this preparation. Application was made to one side only and pain persisted in the untreated side.

## EXAMPLE 4

A severe back pain in a man age 57 weighing 195 lbs. who had multiple prolapsed disc in the lumbar

area causing muscle spasms was relieved by the topical application of 2 milliliters of the preparation described in Example 2 with 20 volume per cent DMSO. The preparation was applied to the skin over the lumbar spine and relieved the pain within one hour for approximately eight hours. The treatments were continued on twelve hour intervals for a period of several days with an occasionally skipped treatment. On some occasions the treatment was not effective but in such cases the subsequent treatment was effective.

The condition being treated had been treated by enzyme injection therapy more than a year previously. Elective surgery was being considered at the time the present treatment was initiated.

#### EXAMPLE 5

Two quarter horses, normally actively engaged in barrel racing, developed tendonitis of the flexor tendons of both hind legs and were successfully treated by topical application of the aqueous sodium hyaluronate solution of Example 2 mixed with a transdermal carrier. The 80 volume percent hyaluronate/20 volume percent dimethyl sulfoxide mixture was more effective than a commercially available topical agent, Absorbine. One leg of each horse was treated with hyaluronate mixture and the other was treated with the Absorbine. The hyaluronate was applied twice each day for three days using three milliliter applications the first day and one milliliter application the subsequent two days (in each case 1/2 of each application was applied on each side of the leg). At the end of three days the symptoms, swelling over the tendon and sheath and soreness over the sesamoid bones, were eliminated. The primary lesion was gone. On the other hand, treatment with Absorbine in the recommended manner required six days of treatment for complete relief.

#### COMPARATIVE EXAMPLE 1

Two experiments were conducted which demonstrated a limitation in the remote administration of hyaluronic acid. Two of the control horses from the study reported in Example 1 were treated topically and two others were treated intravenously after their joint traumas had become chronic and no significant improvement in their conditions was observed. Although some relief of a chronic condition induced by Freund's Complete Adjuvant has been reported for the intra-articular injection of sodium hyaluronate in European Published Patent Application 144,019 this model is commonly restricted to the evaluation of the treatment of acute conditions. The lesions developed an extended period after traumatization are so severe that they are not normally expected to respond to treatment. Thus, these results are not judged to be a major limitation on remote administration and may, in fact, only be reflective of the severity of the induced trauma.

The topical treatment was given on a daily basis for six days beginning on "day 28" of the Example 1 study, i.e. thirty-four days after the induction of trauma, by the application of ten milliliters of a 80 volume percent hyaluronate solution/20 volume percent dimethyl sulfoxide mixture to the affected joint. The aqueous hyaluronate solution was the same as utilized in Example 2. No significant improvement in any of the four criteria of Example 1 were observed as compared to either the immediate pretreatment condition or the other two control horses.

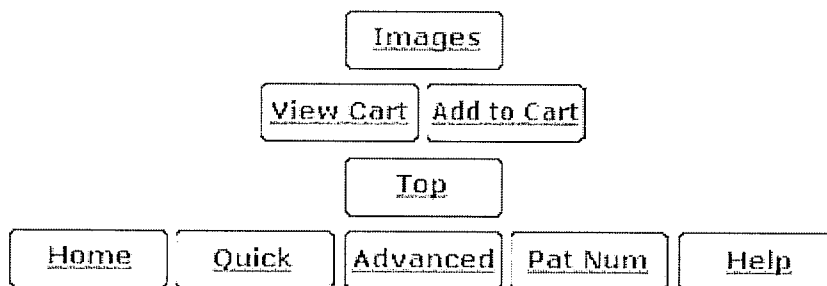
The intravenous treatment was given every other day for eight days beginning on "day 35" of the Example 1 study, i.e. forty-one days after the induction of trauma, by injection into the jugular vein of four milliliters of the aqueous sodium hyaluronate solution used in Example 1. Once again no significant improvement in any of the four criteria was observed.

Although the invention has been described in detail in the foregoing for the purpose of illustration, it is to be understood that such detail is solely for that purpose and that variations can be made therein by

those skilled in the art without departing from the spirit and scope of the invention except as it may be limited by the claims.

\* \* \* \* \*

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# EXHIBIT I

## LEGEND<sup>®</sup> LABEL

*(Legend<sup>®</sup> Injectable Solution (p. 3 of 4); Legend<sup>®</sup>  
Multi Dose Injectable Solution (p. 3 of 6))*

Your Search Terms: PATENT BAYER

Version 1 - Published Jan 04, 2010

**LEGEND - hyaluronate sodium injection, solution**

Bayer HealthCare LLC Animal Health Division

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**Legend®**

**(hyaluronate sodium)**

**Injectable Solution**

**For Intravenous Use**

**In Horses Only**

**CAUTION:**

Federal law restricts this drug to use by or on the order of a licensed veterinarian.

**DESCRIPTION:**

Legend® (hyaluronate sodium) Injectable Solution is a clear, colorless solution of low viscosity. Legend Injectable Solution is pyrogen free, sterile and does not contain a preservative. It is administered by intravenous injection.

Hyaluronic acid, the conjugate acid of hyaluronate sodium, is extracted from the capsule of *Streptococcus* spp. and purified, resulting in a form which is essentially free of protein and nucleic acids.

Legend Injectable Solution is supplied in 4 mL (40 mg) vials. Each mL contains 10 mg hyaluronate sodium, 8.5 mg sodium chloride, 0.223 mg sodium phosphate dibasic, and 0.04 mg sodium phosphate monobasic. The pH is adjusted to between 6.5 and 8.0 with sodium hydroxide or hydrochloric acid.

**CHEMISTRY**

Hyaluronic acid, a glycosaminoglycan, can exist in the following forms depending upon the chemical environment in which it is found: as the acid, hyaluronic acid; as the sodium salt, sodium hyaluronate (hyaluronate sodium); or as the hyaluronate anion. These terms may be used interchangeably but in all cases, reference is made to the glycosaminoglycan composed of repeating subunits of D-glucuronic acid and N-acetyl-D-glucosamine linked together by glycosidic bonds. Since this product originates from a microbial source, there is no potential for contamination with dermatan or chondroitin sulfate or any other glycosaminoglycan.

**CLINICAL PHARMACOLOGY:**

Hyaluronic acid is a naturally occurring substance present in connective tissue, skin, vitreous humour and the umbilical cord in all mammals. High concentrations of hyaluronic acid are also found in the synovial fluid. It also constitutes the major component of the capsule of certain microorganisms. The hyaluronic acid produced by bacteria is of the same structure and configuration as that found in mammals.

The actual mechanism of action for hyaluronate sodium in the healing of degenerative joint disease is not completely understood. One major function appears to be the regulation of normal cellular constituents. This effect decreases the impact of exudation, enzyme release and subsequent degradation of joint integrity. Additionally, hyaluronate sodium exerts an anti-inflammatory action by inhibiting the movement of granulocytes and macrophages.<sup>1</sup>

Hyaluronate molecules are long chains which form a filter network interspersed with normal cellular fluids. It is widely accepted that injection directly into the joint pouch enhances the healing of inflamed synovium by restoring lubrication of the joint fluid. This further supplements the visco-elastic properties of normal joint fluid.

**INDICATIONS:**

Legend® (hyaluronate sodium) Injectable Solution is indicated in the treatment of joint dysfunction of the carpus or fetlock in horses due to non-infectious synovitis associated with equine osteoarthritis.

**CONTRAINDICATIONS:**

There are no known contraindications for the use of Legend® (hyaluronate sodium) Solution in horses.

**RESIDUE WARNING:**

Do not use in horses intended for human consumption.

**HUMAN WARNINGS:**

Not for use in humans. Keep this and all other drugs out of reach of children.

**ANIMAL SAFETY WARNING:**

**Not for Intra-articular use.**

**PRECAUTIONS:**

Radiographic evaluation should be carried out in cases of acute lameness to ensure that the joint is free from serious fracture.

The safety of Legend Injectable Solution has not been evaluated in breeding stallions or in breeding, pregnant or lactating mares.

**ADVERSE REACTIONS:**

No local or systemic side effects were observed in the field studies using Legend Injectable Solution.

**Post-Approval Experience:** While all adverse reactions are not reported, the following adverse reactions are based on voluntary post-approval reporting for Legend Injectable Solution: Occasional depression, lethargy, and fever.

For medical emergencies or to report adverse reactions, call 1-800-422-9874.

**EFFECTIVENESS:**

Forty-six horses with lameness in either the carpal or fetlock joints were treated intravenously or intra-articularly with Legend Injectable Solution in a well-controlled field study conducted at four locations. One, two or three injections were given based on clinical improvement. Overall clinical improvement was judged as excellent or good in 90% of the cases treated intravenously and 96% of those treated intra-articularly with Legend Injectable Solution.

**ANIMAL SAFETY:**

Legend Injectable Solution was administered to normal horses at one, three and five times the recommended intra-articular dosage of 20 mg and intravenous dosage of 40 mg. Treatments were given once weekly for nine consecutive weeks (three times the maximum duration). No systemic clinical signs were observed nor were there any adverse effects upon hematology or clinical chemistry parameters. A transient, slight to mild post injection swelling of the joint capsule occurred in some of the animals treated intra-articularly with Legend Injectable Solution as it did in the saline treated control horse. No gross or histological lesions were observed in the soft tissues or the surface areas of the treated joint.

**DOSAGE AND ADMINISTRATION:**

4 mL (40 mg) injected intravenously. Treatment may be repeated at weekly intervals for a total of three treatments.

Use aseptic technique and inject slowly into the jugular vein.

Horses should be given stall rest after treatment before gradually resuming normal activity.

Discard any unused portion of the drug and empty vial after opening.

#### HOW SUPPLIED:

Legend Injectable Solution is supplied in a carton of six 4 mL (40 mg) bottles.

#### STORAGE:

Do not store above 40° C (104° F).

#### REFERENCE:

<sup>1</sup>Swanstrom, O.G. 1978. Hyaluronate (hyaluronic acid) and its use, Proc. American Assoc. Equine Pract., 24th annual convention, pp. 345-348.

U.S. Patent No. 4,808,576

For customer service or to obtain product information, including a Material Safety Data Sheet, call 1-800-633-3796.

08710999-017699

Made in U.S.A.

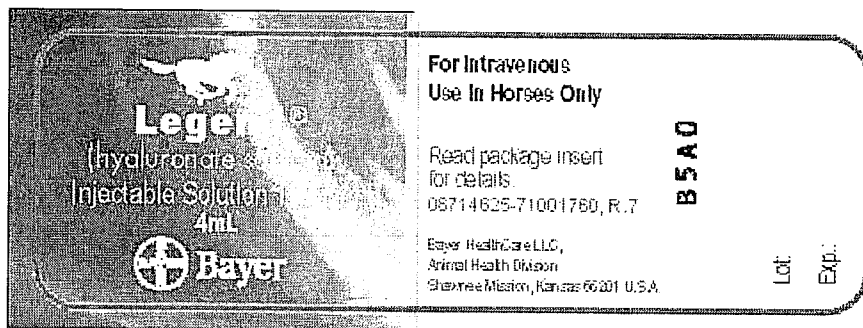
September, 2005

08715524-79001760, R.7

NADA 140-883, Approved by FDA

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**Legend® (hyaluronate sodium) Injectable Solution 10mg/mL 4mL**

For Intravenous Use in Horses Only

Read Package Insert for Details

<b>LEGEND</b>			
hyaluronate sodium injection, solution			
<b>Product Information</b>			
Product Type	PRESCRIPTION ANIMAL DRUG	NDC Product Code (Source)	0859-2272
Route of Administration	INTRAVENOUS	DEA Schedule	
<b>Active Ingredient/Active Moiety</b>			
Ingredient Name	Basis of Strength	Strength	
HYALURONATE SODIUM (HYALURONIC ACID)	HYALURONATE SODIUM	10 mg in 1 mL	
<b>Inactive Ingredients</b>			
Ingredient Name	Strength		
SODIUM CHLORIDE	8.5 mg in 1 mL		
SODIUM PHOSPHATE, DIBASIC	0.223 mg in 1 mL		
SODIUM PHOSPHATE, MONOBASIC	0.04 mg in 1 mL		
<b>Product Characteristics</b>			
Color	Score		
Shape	Size		
Flavor	Imprint Code		
Contains			
<b>Packaging</b>			
# NDC	Package Description	Multilevel Packaging	
1 0859-2272-02	4 mL In 1 BOTTLE	None	

**Marketing Information**

Marketing Category	Application Number or Monograph Citation	Marketing Start Date	Marketing End Date
NADA	NADA140883	09/12/1991	

**Labeler** - Bayer HealthCare LLC Animal Health Division (152266193)

Revised: 05/2009

Bayer HealthCare LLC Animal Health Division

Your Search Terms: PATENT BAYER

Version 1 - Published Jan 04, 2010

**LEGEND - hyaluronate sodium injection, solution**

Bayer HealthCare LLC Animal Health Division

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**Legend® Multi Dose  
(hyaluronate sodium)  
Injectable Solution**

**For Intravenous Use in Horses Only**

**Not for Intra-Articular Use**

**CAUTION:**

Federal law restricts this drug to use by or on the order of a licensed veterinarian.

**DESCRIPTION:**

Legend® Multi Dose (hyaluronate sodium) Injectable Solution is a clear, colorless solution of low viscosity. Legend Multi Dose Injectable Solution is pyrogen free and sterile. It is administered by intravenous injection.

Hyaluronic acid, the conjugate acid of hyaluronate sodium, is extracted from the capsule of *Streptococcus* spp. and purified, resulting in a form which is essentially free of protein and nucleic acids.

Legend Multi Dose Injectable Solution is supplied in 20 mL vials. Each mL contains 10 mg hyaluronate sodium, 8.5 mg sodium chloride, 0.223 mg sodium phosphate dibasic, 0.04 mg sodium phosphate monobasic and 15.63 mg benzyl alcohol as a preservative. The pH is adjusted to between 6.5 and 8.0 with sodium hydroxide or hydrochloric acid.

**CHEMISTRY:**

Hyaluronic acid, a glycosaminoglycan, can exist in the following forms depending upon the chemical environment in which it is found: as the acid, hyaluronic acid; as the sodium salt, sodium hyaluronate (hyaluronate sodium); or as the hyaluronate anion. These terms may be used interchangeably but in all cases, reference is made to the glycosaminoglycan composed of repeating subunits of D-glucuronic acid and N-acetyl-D-glucosamine linked together by glycosidic bonds. Since this product originates from a microbial source, there is no potential for contamination with dermatan or chondroitin sulfate or any other glycosaminoglycan.

**CLINICAL PHARMACOLOGY:**

Hyaluronic acid is a naturally occurring substance present in connective tissue, skin, vitreous humour and the umbilical cord in all mammals. High concentrations of hyaluronic acid are also found in the synovial fluid. It also constitutes the major component of the capsule of certain microorganisms. The hyaluronic acid produced by bacteria is of the same structure and configuration as that found in mammals.

The actual mechanism of action for hyaluronate sodium in the healing of degenerative joint disease is not completely understood. One major function appears to be the regulation of normal cellular constituents. This effect decreases the impact of exudation, enzyme release and subsequent degradation of joint integrity. Additionally, hyaluronate sodium exerts an antiinflammatory action by inhibiting the movement of granulocytes and macrophages.<sup>1</sup> Hyaluronate molecules are long chains which form a filter network interspersed with normal cellular fluids.

**INDICATIONS:**

Legend Multi Dose (hyaluronate sodium) Injectable Solution is indicated in the treatment of joint dysfunction of the carpus or fetlock in horses due to non-infectious synovitis associated with equine osteoarthritis.

**CONTRAINDICATIONS:**

There are no known contraindications for the use of Legend Multi Dose Injectable Solution in horses.

**RESIDUE WARNING:**

Do not use in horses intended for human consumption.

**HUMAN WARNINGS:**

Not for use in humans. Keep this and all other drugs out of reach of children.

**ANIMAL SAFETY WARNINGS:**

**Not for Intra-articular use. The Intra-articular safety of hyaluronate sodium with benzyl alcohol has not been evaluated.**

**PRECAUTIONS:**

Radiographic evaluation should be carried out in cases of acute lameness to ensure that the joint is free from serious fracture.

The safety of Legend Multi Dose Injectable Solution has not been evaluated in breeding stallions or in breeding, pregnant or lactating mares.

**ADVERSE REACTIONS:**

No local or systemic side effects were observed in the field studies using Legend Injectable Solution.

**Post-Approval Experience:** While all adverse reactions are not reported, the following adverse reactions are based on voluntary post-approval reporting for Legend Injectable Solution: Occasional depression, lethargy, and fever.

For medical emergencies or to report adverse reactions, call 1-800-422-9874.

**EFFECTIVENESS:**

Effectiveness studies utilizing Legend Multi Dose Injectable Solution were not performed. Legend Multi Dose Injectable Solution was approved based on the conclusion that the effectiveness of Legend Multi Dose Injectable Solution will not differ from that demonstrated for the original formulation of Legend Injectable Solution.

Twenty-one horses with lameness in either the carpal or fetlock joints were treated intravenously with Legend Injectable Solution in a well-controlled field study conducted at four locations. One, two or three injections were given based on clinical improvement. Overall clinical improvement was judged as excellent or good in 90% of the cases treated intravenously with Legend Injectable Solution.

**ANIMAL SAFETY:**

Animal safety studies utilizing Legend Multi Dose Injectable Solution were not performed. Legend Multi Dose Injectable Solution was approved based on the conclusion that the safety of Legend Multi Dose Injectable Solution will not differ from that demonstrated for the original formulation of Legend Injectable Solution.

Legend Injectable Solution was administered to normal horses at one, three and five times the recommended intravenous dosage of 40 mg. Treatments were given once weekly for nine consecutive weeks (three times the maximum duration). No systemic clinical signs were observed nor were there any adverse effects upon hematology or clinical chemistry parameters.



**DOSAGE AND ADMINISTRATION:**

4 mL (40 mg) injected intravenously. Treatment may be repeated at weekly intervals for a total of three treatments.

Use aseptic technique and inject slowly into the jugular vein.

Horses should be given stall rest after treatment before gradually resuming normal activity.

**HOW SUPPLIED:**

Legend Multi Dose Injectable Solution is supplied in 20 mL bottles.

**STORAGE:**

Do not store above 40° C (104° F).

**REFERENCE:**

<sup>1</sup>Swanstrom, O.G. 1978. Hyaluronate (hyaluronic acid) and its use, Proc. American Assoc. Equine Pract., 24th annual convention, pp. 345-348.

U.S. Patent No. 4,808,576

For customer service or to obtain product information, including a Material Safety Data Sheet, call 1-800-633-3796.

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Made in U.S.A.

September, 2005

08904793, R.0

NADA 140-883, Approved by FDA

Bayer HealthCare LLC

Animal Health Division

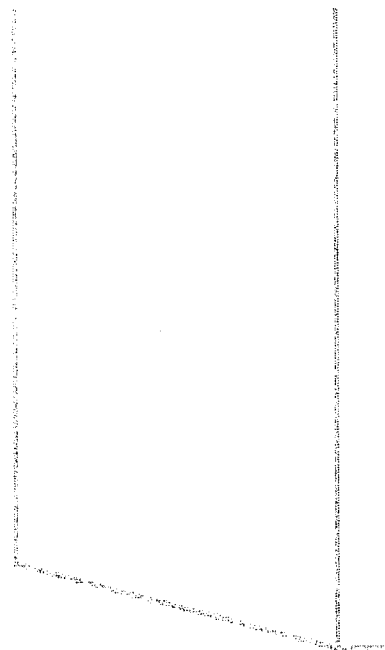
Shawnee Mission, Kansas 66201 U.S.A.

12826



LabelDataPlus - Legend Label Detail

<http://www.labeldataplus.com/detail.php?c=11672>



**Legend Multi Dose (hyaluronate sodium) Injectable Solution 10mg /mL For Intravenous Use in Horses Only 20mL**

<b>LEGEND</b>			
hyaluronate sodium injection, solution			
<b>Product Information</b>			
Product Type	PRESCRIPTION ANIMAL DRUG	NDC Product Code (Source)	0859-2273
Route of Administration	INTRAVENOUS	DEA Schedule	
<b>Active Ingredient/Active Moiety</b>			
Ingredient Name	Basis of Strength	Strength	
HYALURONATE SODIUM (HYALURONIC ACID)	HYALURONATE SODIUM	10 mg in 1 mL	
<b>Inactive Ingredients</b>			
Ingredient Name	Strength		
SODIUM CHLORIDE	8.5 mg in 1 mL		
SODIUM PHOSPHATE, DIBASIC	0.223 mg in 1 mL		
SODIUM PHOSPHATE, MONOBASIC	0.04 mg in 1 mL		
BENZYL ALCOHOL	15.63 mg in 1 mL		
<b>Product Characteristics</b>			
Color	Score		
Shape	Size		
Flavor	Imprint Code		
Contains			
<b>Packaging</b>			
# NDC	Package Description	Multilevel Packaging	
1 0859-2273-01	1 BOTTLE In 1 CARTON	contains a BOTTLE	
1	20 mL In 1 BOTTLE	This package is contained within the CARTON (0859-2273-01)	

**Marketing Information**

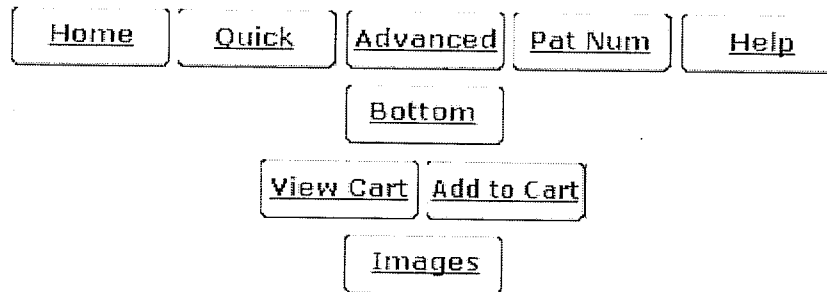
Marketing Category	Application Number or Monograph Citation	Marketing Start Date	Marketing End Date
NADA	NADA140883	09/12/1991	

**Labeler** - Bayer HealthCare LLC Animal Health Division (152266193)

Revised: 05/2009

Bayer HealthCare LLC Animal Health Division

EXHIBIT J  
PATENT 4,530,787

USPTO PATENT FULL-TEXT AND IMAGE DATABASE

( 1 of 1 )

**United States Patent****4,530,787****Shaked , et al.****July 23, 1985**

Controlled oxidation of microbially produced cysteine-containing proteins

**Abstract**

Method of oxidizing reduced cysteine-containing microbially produced synthetic proteins, such as synthetic IFN-.beta. or synthetic IL-2, in a controlled manner so that the synthetic proteins have the same disulfide bridging as their native counterparts. The oxidation employs o-iodosobenzoate as oxidizing agent and is carried out in an aqueous medium at a pH at least about one-half pH unit less than the pK.sub.a of the cysteines to be oxidized, a synthetic protein concentration of less than about 5 mg/ml, and an oxidizing agent:protein mol ratio that is at least stoichiometric, provided that the oxidizing agent is in excess in the terminal portion of the reaction.

Inventors: **Shaked; Ze'ev** (Berkeley, CA), **Wolfe; Sidney N.** (Richmond, CA)Assignee: **Cetus Corporation** (Emeryville, CA)Appl. No.: **06/661,902**Filed: **October 17, 1984****Related U.S. Patent Documents**

<u>Application Number</u>	<u>Filing Date</u>	<u>Patent Number</u>	<u>Issue Date</u>
594351	Mar., 1984		

**Current U.S. Class:**

**530/351** ; 424/85.1; 424/85.2; 424/85.4; 424/85.6; 424/85.7;  
 435/69.51; 435/69.52; 435/811; 530/345; 530/350; 530/363;  
 530/395; 530/399; 530/402; 530/410; 530/808; 530/820;  
 530/825; 930/141; 930/142

**Current International Class:**

C07K 1/00 (20060101); C07K 1/00 (20060101); C07K  
 1/36 (20060101); C07K 1/36 (20060101); C07K  
 1/113 (20060101); C07K 1/113 (20060101); C07K  
 14/435 (20060101); C07K 14/435 (20060101); C07K

14/565 (20060101); C07K 14/565 (20060101); C07K  
14/55 (20060101); C07K 14/55 (20060101); C07G 007/00 ();  
A61K 045/02 ()

**Field of Search:**

260/112.5R, 112R 424/85, 88 435/68, 811

**References Cited [Referenced By]****U.S. Patent Documents**

<u>4061538</u>	December 1977	Dorner et al.
<u>4411993</u>	October 1983	Gillis
<u>4414147</u>	November 1983	Klibanov et al.
<u>4432895</u>	February 1984	Tarnowski
<u>4450103</u>	May 1984	Konrad
<u>4462940</u>	July 1984	Hanisch et al.
<u>4478744</u>	October 1984	Mezei et al.

**Other References**

Tietze, Anal. Biochem., (1969), 27: 502-522. .  
Hellerman et al., J. Am. Chem. Soc., (1941), 63: 2551-2553. .  
Chinard et al., Methods Biochem. Anal., (1954), 1: 1-26. .  
Vallejos et al., FEBS Letters, (1976), 61: 95-99. .  
Guzman Barron, Advan. Enzymol., (1951), 11: 223-226. .  
Liu, The Proteins, (1978), vol. III, 255-263, Academic Press..

*Primary Examiner:* Schain; Howard E.

*Attorney, Agent or Firm:* Halluin; Albert P. Hasak; Janet E.

**Parent Case Text**

This application is a continuation-in-part application of copending U.S. application Ser. No. 594,351 filed Mar. 28, 1984, now abandoned.

**Claims**

What is claimed is:

1. A preparative process for oxidizing a microbially produced synthetic protein having fully reduced cysteines and having an amino acid sequence substantially identical to a useful protein which sequence includes cysteines which in the useful protein are linked intramolecularly to form a cystine in a controlled manner whereby said cysteines are oxidized selectively to form said cystine with minimal overoxidation and formation of nonconforming cysteine groups or oligomers comprising reacting the fully reduced microbially produced synthetic protein with o-iodosobenzoate in an aqueous medium at a pH at least about one-half pH unit below the pK<sub>sub.a</sub> of said cysteines and wherein the concentration of synthetic protein in the reaction mixture is less than about 5 mg/ml and the mol ratio of o-



iodosobenzoate to protein is at least stoichiometric, with the proviso that the o-iodosobenzoate is in excess in the terminal portion of the reaction.

2. The process of claim 1 wherein the useful protein is a native protein having useful biological activity and the intramolecular linking is essential to the biological activity or enhances the biological activity.
3. The process of claim 1 wherein the protein is a lymphokine.
4. The process of claim 1 wherein the protein is IFN-.beta. or IL-2.
5. The process of claim 4 wherein the pH is below about 9.
6. The process of claim 4 wherein the pH is between 5.5 and 9.
7. The process of claim 1 wherein the protein is IL-2 and the pH is between 6.5 and 7.5.
8. The process of claim 1 wherein the protein is IFN-.beta. and the pH is between 6.5 and 9.0.
9. The process of claim 1 wherein said concentration of synthetic protein is in the range of about 0.3 to about 0.7 mg/ml.
10. The process of claim 1 wherein the protein is an IL-2, said mol ratio is in the range of about 1:1 and about 5:1, the pH is between 6.5 and 7.5, and the concentration of synthetic IL-2 in the reaction mixture is in the range of about 0.3 to about 0.7 mg/ml.
11. The process of claim 1 wherein the protein is IFN-.beta., said mol ratio is in the range of about 1:1 and about 5:1, the pH is between 6.5 and 9.0 and the concentration of synthetic IFN-.beta. in the reaction mixture is in the range of about 0.3 to about 0.7 mg/ml.
12. The process of claim 1 wherein after said oxidation the oxidized product is purified using a gel filtration method.
13. The process of claim 12 wherein the filtration is carried out using a G-25 Sephadex desalting column.
14. The preparation of claim 1 wherein the preparation contains less than about 1% by weight oligomers.
15. The preparation of claim 1 wherein the synthetic protein is a synthetic mutein of a biologically active protein which protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutein having at least one of said cysteine residues deleted or replaced by another amino acid.
16. A cystine-containing IL-2 preparation derived from synthetic microbially produced IL-2 having fully reduced cysteines comprising cystine-containing IL-2 which:
  - (i) has the same disulfide bridging as native human IL-2;
  - (ii) is substantially free of oligomers; and
  - (iii) contains less than about 15% by weight of isomers having disulfide bridging different from native

human IL-2.

17. A cystine-containing protein preparation derived from a synthetic microbially produced, unglycosylated protein having fully reduced cysteines and having an amino acid sequence substantially identical to a useful protein which sequence includes cysteines which is the useful protein are linked intramolecularly to form a cystine, which preparation consists essentially of a cystine-containing protein which:

(i) has the same disulfide bridging as the native useful protein;

(ii) is substantially free of oligomers; and

(iii) contains less than about 15% by weight of isomers having disulfide bridging different from the native useful protein.

18. The preparation of claim 16 wherein the preparation contains less than about 1% by weight oligomers.

19. A cystine-containing IFN-.beta. preparation derived from synthetic microbially produced, unglycosylated IFN-.beta. having fully reduced cysteines, which preparation consists essentially of cystine-containing IFN-.beta. which:

(i) has the same disulfide bridging as native human IFN-.beta.;

(ii) is substantially free of oligomers; and

(iii) contains less than about 15% by weight of isomers having disulfide bridging different from native human IFN-.beta..

20. The preparation of claim 19 wherein the preparation contains less than about 1% by weight oligomers.

21. The preparation of claim 16 wherein said IL-2 is des-ala IL-2.sub.ser125.

22. The preparation of claim 19 wherein said IFN-.beta. is IFN-.beta..sub.ser17.

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*Description*

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## BACKGROUND OF THE INVENTION

### 1. Technical Field

This invention is in the field of biochemical engineering. More particularly, it concerns a method of oxidizing fully reduced cysteine-containing microbially produced proteins in a controlled manner so that they have disulfide bridging identical to their naturally occurring counterparts.

### 2. Background Art

When active proteins that contain one or more disulfide bridges are produced microbially via genetic

engineering techniques, the synthetic protein is made by the microorganism in a reduced form lacking disulfide bridging or in the form of oligomers that are made in the cell by uncontrolled thiol-disulfide interchange reactions. Tietze, F., *Anal Biochem* (1969) 27:502. If it is desirable or necessary that the synthetic protein have the same primary structure as its native counterpart, the biochemical engineer is faced not only with the problem of separating the protein from the microorganism culture, but also the problems of reducing oligomers and/or oxidizing the reduced synthetic protein so that it assumes the primary structure of the native protein. Previous oxidations of synthetic microbially produced proteins have been uncontrolled and done deliberately by subjecting the protein to oxidizing conditions or incidentally by placing the proteins in an environment in which it is oxidized. Oxidizing the protein in an uncontrolled manner may: result in the formation of undesirable isomers (incorrect intramolecular bridging) or polymers (intermolecular bridging); overoxidation; complicate the separation of the protein from the culture, or reduce the yield of protein having the desired primary structure. In the case of proteins that are intended for therapeutic use, uncontrolled oxidation through purification, formulation or administration yields a nonhomogeneous material that is contaminated with isomers and/or oligomers that may be inactive or antigenic.

The present invention is directed to a process for oxidizing such microbially produced proteins in a selective, controlled manner using an oxidizing agent, preferably o-iodosobenzoic acid, that oxidizes cysteines selectively, such that the desired disulfide bridging is produced in high yield. In this regard, o-iodosobenzoic acid is a well known sulfhydryl reagent that has been used previously to oxidize vicinal cysteines of native proteins selectively. Hellerman, L., et al., *J Amer Chem Soc* (1941) 63:2551-2552, Chinard, F. P. and Hellerman, L., *Methods Biochem Anal* (1954) 1:1, and Vallejos, R. H. and Andreo, C. S., *FEBS Letters* (1976) 61:95-99. Other oxidizing agents for thiol groups in native proteins are described by Guzman Barron, E. S., *Advan Enzymol* (1951) 11:223-226 and Teh-Yung Liu, *The Proteins* (1978) Vol III, 255-263, Academic Press, N.Y. To the best of applicants' knowledge, prior use of o-iodosobenzoic acid and other oxidizing agents as selective oxidants for sulfhydryl groups in proteins has been for analytical purposes. Applicants know of no prior art concerning the use of such oxidants in preparative processes to carry out controlled oxidation of synthetic microbially produced proteins.

## SUMMARY OF THE INVENTION

The invention is a preparative process for oxidizing a fully reduced microbially produced synthetic protein having an amino acid sequence substantially identical to a useful protein which sequence includes cysteines which in the useful protein are linked intramolecularly to form a cystine in a controlled manner whereby said cysteines are oxidized selectively to form said cystine with minimal overoxidation and formation of nonconforming cysteine groups or oligomers comprising reacting the fully reduced microbially produced synthetic protein with an o-iodosobenzoate in an aqueous medium at a pH at least about one-half pH unit lower than the  $pK_{sub.a}$  of said cysteines and wherein the concentration of synthetic protein in the reaction mixture is less than about 5 mg/ml and the mol ratio of o-iodosobenzoate to protein is at least stoichiometric, with the proviso that the o-iodosobenzoate is in excess in the terminal portion of the reaction.

Also part of this invention are novel oxidized preparations produced by the above-described controlled oxidation of synthetic proteins having an amino acid sequence substantially identical to a useful protein which sequence includes cysteines which in the useful protein are linked intramolecularly to form a cystine. Preferably the protein is a mutein as defined further below or microbially produced IFN- $\beta$  or IL-2. These preparations comprise a synthetic protein that (a) has the same disulfide bridging as its native counterpart, (b) is substantially free of oligomers and (3) contains less than about 15% of isomers having disulfide bridging different from its native counterpart.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The synthetic proteins that are oxidized by the invention are exogenous to the genetically engineered microorganisms that produce them. They have amino acid sequences that are substantially identical to useful proteins and include cysteine residues which in the useful protein are linked intramolecularly to form one or more cystine (intrapeptidial disulfide bridges) moieties. In this regard the term "substantially identical" means that the amino acid sequences of the synthetic and useful proteins are either identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not cause an adverse functional dissimilarity between the synthetic protein and its nonmicrobially produced counterpart. The synthetic proteins that are oxidized in the invention process are fully reduced, i.e., they lack disulfide bridging. If the protein is produced by the microorganism in an oxidized form it must be reduced before being subjected to the oxidation. Reduction may be accomplished by treating the protein with a reducing agent such as dithiothreitol or 2-mercaptoethanol.

Synthetic proteins of particular interest are those that have amino acid sequences that are substantially identical to native proteins having useful biological activity and disulfide bridging that is essential to such activity or enhances such activity. Examples of such native proteins are lymphokines such as interferon-beta (IFN-.beta.), the interferon-alphas (IFN-.alpha.), interleukin-2 (IL-2), and colony stimulating factor-1.

Also of particular interest are synthetic proteins which are muteins of biologically active proteins in which at least one cysteine residue that is not essential to biological activity, that is present in the biologically active protein and that is free to form a disulfide link has been deliberately deleted or replaced with another amino acid to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation.

Proteins which may be mutationally altered in this manner may be identified from available information regarding the cysteine content of biologically active proteins and the roles played by the cysteine residues with respect to activity and tertiary structure. For proteins for which such information is not available in the literature, this information may be determined by systematically altering each of the cysteine residues of the protein by the procedures described herein and testing the biological activity of the resulting muteins and their proclivity to form undesirable intermolecular or intramolecular disulfide bonds. Accordingly, while the invention is specifically exemplified below as regards muteins of IFN-.beta. and IL-2, it will be appreciated that the following teachings apply to any other biologically active protein that contains a functionally nonessential cysteine residue that makes the protein susceptible to undesirable disulfide bond formation. Examples of proteins other than IFN-.beta. and IL-2 that are candidates for mutational alteration according to the invention are lymphotoxin (tumor necrosis factor), colony stimulating factor-1, and IFN-.alpha.1. Candidate proteins will usually have an odd number of cysteine residues.

In the case of IFN-.beta. it has been reported in the literature that both the glycosylated and unglycosylated IFNs show qualitatively similar specific activities and that, therefore, the glycosyl moieties are not involved in and do not contribute to the biological activity of IFN-.beta.. However, bacterially produced IFN-.beta. which is unglycosylated consistently exhibits quantitatively lower specific activity than native IFN-.beta. which is glycosylated. IFN-.beta. is known to have three cysteine residues at positions 17, 31 and 141. Cysteine 141 has been demonstrated by Shepard, et al., supra, to be essential for biological activity. In IFN-.alpha., which contains four cysteine residues, there are two intramolecular --S--S-- bonds: one between cys 29 and cys 138 and another between cys 1 and cys 98. Based on the homology between IFN-.beta. and IFN-.alpha.s cys 141 of IFN-.beta. could be involved in an intramolecular --S--S-- bond with cys 31, leaving cys 17 free to form intermolecular crosslinks. By either deleting cys 17 or substituting it by a different amino acid, one can determine whether cys 17 is



essential to biological activity, and its role in --SS-- bond formation. If cys 17 is not essential for the biological activity of the protein, the resulting cys 17-deleted or cys 17-substituted protein might exhibit specific activity close to that of native IFN-.beta. and would possibly also facilitate isolation and purification of the protein.

By the use of the oligonucleotide-directed mutagenesis procedure with a synthetic oligonucleotide primer that is complementary to the region of the IFN-.beta. gene at the codon for cys 17 but which contains single or multiple base changes in that codon, a designer gene may be produced that results in cys 17 being replaced with any other amino acid of choice. When deletion is desired the oligonucleotide primer lacks the codon for cys 17. Conversion of cys 17 to neutral amino acids such as glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine is the preferred approach. Serine and threonine are the most preferred replacements because of their chemical analogy to cysteine. When the cysteine is deleted, the mature mutein is one amino acid shorter than the native parent protein or the microbially produced IFN-.beta..

Human IL-2 is reported to have three cysteine residues located at positions 58, 105, and 125 of the protein. As in the case of IFN-.beta., IL-2 is in an aggregated oligomeric form when isolated from bacterial cells and has to be reduced with reducing agents in order to obtain a good yield from bacterial extracts. In addition, the purified reduced IL-2 protein is unstable and readily reoxidized upon storage to an oligomeric inactive form. The presence of three cysteines means that upon reoxidation, the protein may randomly form one of three possible intramolecular disulfide bridges, with only one of those being the correct bridge as found in the native molecule. Since the disulfide structure of the native IL-2 protein is not known, it is possible to use the present invention to create mutations at codons 58, 105 and 125 of the IL-2 gene and identify which cysteine residues are necessary for activity and therefore most likely to be involved in native disulfide bridge formation. In the same vein, the cysteine residue that is not necessary or activity can be modified so as to prevent the formation of incorrect intramolecular disulfide bridges and minimize the chance of intermolecular disulfide bridges by removal or replacement of the free cysteine residue.

The synthetic counterparts, including the above-described muteins, of the native proteins which may be oxidized are made by genetic engineering techniques. These techniques typically involve identifying and characterizing the structural gene that encodes the native protein, isolating or synthesizing that gene or a mutant that encodes a functionally equivalent mutein of the native protein, inserting the gene into an appropriate expression vector in a position that permits expression of the gene, transforming competent microorganisms with the vector, identifying correct transformants, and culturing the transformants in a suitable growth medium. The protein is typically recovered from the culture by disrupting the cells, treating the disruptate with solubilizing agents (depending on the solubility characteristics of the protein) and one or more extractants to isolate crude protein, and purifying the crude protein by various preparative chromatography procedures. If the protein is produced by the microorganisms in oligomeric form or is susceptible to oligomer formation during the recovery, the protein will be treated with a reducing agent at an appropriate stage in the recovery process.

After the synthetic protein is recovered from the microorganism in a crude, substantially pure, or pure form, it is reduced, if necessary, and then oxidized in a controlled manner using the invention process. Controlled oxidation pursuant to the invention process causes the formation of disulfide bridging in the synthetic protein that conforms to the bridging in its native counterpart with no or minimal overoxidation and formation of nonconforming bridging or oligomers. Such oxidation enables the production of high yields of the synthetic protein in a configuration that most closely resembles the configuration of its native counterpart, thereby ensuring the likelihood that the synthetic protein will be functionally equivalent to the native protein.

The oxidant (o-iodosobenzoate) that is used in the process oxidizes cysteine residues selectively and stoichiometrically. In this regard, the term "selectively" indicates that the oxidant (1) oxidizes the cysteines to the disulfide level with no or insignificant oxidation to higher levels and (2) preferentially oxidizes active cysteines that are positioned proximally in the reduced protein. The mol ratio of oxidant to synthetic protein may vary widely depending on the oxidant used. The mol ratio will be at least stoichiometric (1:1 or greater) and will typically be in the range of 1:1 to 100:1. In the case of o-iodosobenzoate, the mol ratio will usually be in the range of about 1:1 to about 5:1. In all instances, the oxidant is in excess during the terminal portion of the reaction to ensure complete oxidation of the reduced protein. These conditions may be achieved by running the reaction with excess oxidant over its entire duration or running the reaction with approximately equimolar portions of reactants over the majority of the reaction period and adding excess oxidant near the end of the reaction period. If the protein is particularly susceptible to oligomerization it is preferable to use reactant proportions that effect pseudo first order kinetics for the oxidant. Such kinetics occur when the oxidant is present in slight excess within the above-mentioned mol ratio range. The concentration of protein in the reaction mixture is kept low, i.e., less than about 5 mg/ml, usually about 0.1 to about 1.5 mg/ml, and preferably about 0.3 to about 0.7 mg/ml, in order to reduce the likelihood of oligomer formation.

The pH of the reaction medium is maintained at a level at least about one-half pH unit below the pK<sub>a</sub> of the cysteine residues being oxidized. When the pK<sub>a</sub>s of these residues differ, the pH is preferably maintained at least about one-half pH unit less than the cysteine residue having the lowest pK<sub>a</sub>. Control of the pH in this manner controls the amount of nonionized thiol, thereby controlling the rate of the reaction and favoring the formation of the desired disulfide bridging. Use of pHs significantly above the specified pH may cause increased production of undesired isomers and oligomers. Excessively high pHs, i.e., greater than about 9, may result in increased oligomer formation and are, therefore, not recommended in most instances. For synthetic IFN- $\beta$ , the pH is maintained between 6 and 9, preferably 6.5 and 8.0. For synthetic IL-2, it is maintained between 5.5 and 9, preferably 7.0 and 8.0.

Thiol pK<sub>a</sub> values may be determined by the procedures described by Irving, R. J., et al., *Acta Chemica Scandinavica* (1964) 18:769-787; Shaked, Z., et al, *Biochemistry* (1981) 19:4256-4266; and Snyder, G. H., et al., *Biochemistry* (1981) 20:6509-6519 and the desired pH range for a given synthetic polypeptide calculated from such determinations. Alternatively, operable and preferred pH ranges for oxidizing a given synthetic protein may be determined empirically.

The oxidation reaction time will depend upon the volume of the reaction mixture. The reaction temperature is not critical and will normally be between 20.degree. C. and 25.degree. C., conveniently room temperature. The oxidation reaction may be terminated by lowering the pH to a level at which the reaction ceases (about pH 4.5). Following the reaction, residual oxidizing agent and undesired isomers and oligomers may be removed chromatographically. If necessary, the oxidized protein may be purified further using protein purification procedures such as gel filtration, high performance liquid chromatography, followed by diafiltration or the like.

In one preferred purification technique for the oxidized protein, small molecular weight species such as sodium dodecyl sulfate or the o-iodosobenzoic acid are removed from the protein pool using gel filtration, for example, a Sephadex G-25 desalting column, rather than diafiltration. Such a gel filtration process generally represents a simple, rapid, reliable, mild, high-recovery process for the purification. For purifying interferon- $\beta$  and IL-2 the G-25 desalting column step may be used to remove the solubilizing detergent SDS which is present during oxidation. For IFN- $\beta$ , an alkaline environment of 10 mM sodium hydroxide is generally required due to the insolubility of the interferon- $\beta$  in neutral solutions of pH 6-8. With diafiltration the interferon is subjected to an alkaline pH 12 environment for as long as 4-5 hours so that heterogeneity is introduced into the sample. Gel filtration reduces the total

incubation time at pH 12 to only 20-70 minutes depending on the flow rate. In addition, it is possible to run the G-25 desalting column at lower pH values of 10.3-11. These two improvements eliminate heterogeneity which has been observed in the post-diafiltered interferon-beta. The only disadvantage of using gel filtration, dilution of the protein, can be controlled by optimizing the sample loading and by choosing the smallest grade of particle size. Furthermore, in most processes gel filtration is not the final step so that concentration or a further dilution of the sample occurs.

The preparation produced by the controlled oxidation consists essentially of synthetic protein having the disulfide bridging of its native counterpart. It is substantially free of oligomers (less than about 1% by weight) and contains less than about 15% by weight isomers having disulfide bridging different from the native counterpart. Synthetic proteins that have been designed to eliminate the possibility of isomer formation (e.g., IL-2 in which the cysteine at position 125 has been changed to serine or IFN-.beta. in which the cysteine at position 17 has been changed to serine), of course, contain no isomers. In contrast, preparations made via uncontrolled oxidations typically contain significant amounts of oligomers (5%-10%) and much larger amounts of undesired isomers. In the case of IL-2 and IFN-.beta., the oxidized proteins are more water soluble than the reduced species. Accordingly, the solubilizing agent (e.g., SDS) may be substantially removed from the preparation, leaving a purified product that is sufficiently water soluble to permit formulation with conventional aqueous parenteral vehicles.

Since the preparations prepared by the controlled oxidization contain more desired product and fewer contaminants than preparations made via uncontrolled oxidation, they may be less antigenic and will usually be more active. Preparations of therapeutic proteins will comprise a therapeutically effective amount of the protein in admixture with a pharmaceutically acceptable carrier. In the case of IFN-.beta. and IL-2, the preparation will usually be formulated for parenteral administration in aqueous vehicles such as distilled water, Ringer's solution, Hank's solution, and physiological saline. IFN-.beta. will usually be administered to humans at doses in the range of 1.times.10.sup.5 to 4.times.10.sup.8 units, whereas IL-2 will usually be administered at about 1.times.10.sup.4 to 2.times.10.sup.8 units.

The following examples further illustrate the invention process. These examples are not intended to limit the invention in any manner. In these examples all temperatures are in degrees Celsius unless otherwise indicated.

#### EXAMPLE 1

##### Controlled Oxidation of IFN-.beta..sub.ser17

##### Preparation of Fully-Reduced IFN-.beta..sub.ser17

IFN-.beta..sub.ser17 is a microbially produced mutein of IFN-.beta. in which the cysteine residue at amino acid position 17 is replaced with a serine residue. IFN-.beta..sub.ser17 has two remaining cysteine residues: one at position 31 and the other at position 141. In native IFN-.beta. the cysteines at positions 31 and 141 interact to form a disulfide bridge. The genetically engineered E. coli microorganism strain used in this example to produce IFN-.beta..sub.ser17 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 USA on Nov. 18, 1983 under accession number 39,517.

These genetically engineered E. coli were grown in the following medium:

	Approximate Initial Ingredient Concentration
	Na.sub.3 Citrate.2H.sub.2 O 3 mM KH.sub.2 PO.sub.4
30 mM (NH.sub.4).sub.2 SO.sub.4 74 mM MgSO.sub.4.7H.sub.2 O 3 mM MnSO.sub.4.H.sub.2 O	



46 .mu.M ZnSO.sub.4.7H.sub.2 O 46 .mu.M CuSO.sub.4.5H.sub.2 O 1-2 .mu.M L-tryptophan  
350 .mu.M FeSO.sub.4.7H.sub.2 O 74 .mu.M thiamine.HCl 0.002% glucose 0.5%

Dow Corning Antifoam B, 25% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

Temperature was maintained at 37 .+-.1.degree. C., pH at 6.5.+-.0.1 with NaOH, and dissolved oxygen at 30% of air saturation. Optical density and residual glucose measurements were taken at 14 hours and at approximately one hour intervals thereafter. Harvest was made when glucose consumption reached 40.+-.6 g/l (OD at 680 nM=10-11).

The harvested material was concentrated approximately 3-fold by circulating it through a microporous cross-flow filter under pressure. The concentrated cells were diafiltered against deionized water until the harvest material was concentrated 4-5 fold. The cells were then disrupted by passing them through a Manton-Gaulin homogenizer at 4.1-5.5.times.10.sup.4 kpa. After the initial pass sodium dodecyl sulfate (SDS)-sodium phosphate buffer was added to a final concentration of 2% SDS, 0.08M sodium phosphate, and homogenization was continued for one hour. Solid dithiothreitol (DTT) was then added to a final concentration of 50 mM and the homogenizate was heated to 90.+-.5.degree. C. for 10 minutes. The resulting cell suspension was extracted with 2-butanol at a 1:1 2-butanol:suspension volume ratio in a static mixer. The mixture was then centrifuged and the 2-butanol rich phase was collected.

The 2-butanol rich phase was mixed with 2.5 volumes of 0.1% SDS in phosphate buffered saline (PBS). Solid DTT was added to a final concentration of 1 mM. The pH of the mixture was adjusted to 6.2.+-.0.1 with glacial acetic acid and this mixture was centrifuged. The resulting paste was collected and resuspended in PBS +10% SDS with pH adjustment to 8.5.+-.0.1 using 1N NaOH. Solid DTT was added to a final concentration of 100 mM and the suspension was heated to 90.+-.5.degree. C. for 10 minutes. The suspension was then cooled to about 25.degree. C., the pH was adjusted to 5.5.+-.0.1 with glacial acetic acid, and the solution was filtered.

The solution was then applied to a Sephacryl S-200 precolumn and the fractions containing highest interferon activities were pooled and concentrated by ultrafiltration with a 10 Kdal molecular weight cut-off.

#### Oxidation of Fully Reduced IFN-.beta..sub.ser17

A 1 mM o-iodosobenzoic acid solution was prepared by mixing the acid in water, sonicating the mixture for about 5 minutes and then stirring and adding 2% NaOH slowly to obtain a final pH of 8.2.+-.0.2 (additional sonication may be used as an alternative to adding base).

A reaction buffer medium was prepared by dissolving Na.sub.4 P.sub.2 O.sub.7.10H.sub.2 O in water to a concentration of 2 mM. The pH of this solution was adjusted to 9.0 by adding 10% acetic acid. SDS to 0.1%, ethylenediaminetetraacetic acid (EDTA) to 1mM and the o-iodosobenzoic acid solution to 15.times.10.sup.-6 M were added to the solution.

The buffer medium was placed in a reaction vessel equipped with a magnetic stirrer and a pH electrode set at 9.0. The IFN-.beta..sub.ser17 preparation and the o-iodosobenzoic acid solutions were added to the reaction mixture from holding vessels using peristaltic pumps that were calibrated to introduce equivalent mol ratios of the IFN and oxidizing agent. The pH of the reaction mixture was controlled at 9.0 by adding 0.25M NaOH via a peristaltic pump at 5 ml/hr. as needed. The IFN-.beta. solution (5 mg/ml in 50 mM acetate buffer, pH 5.5) was added at a flow rate of 2 ml/hr. (7.0 micromole/hr.) for

about 5 hours; the o-iodosobenzoic acid solution was added at 7 ml/hr. (7 micromole/hr.) over the same time period. The addition of the acid solution was continued thereafter to get a final excess of 10-15 mol. The reaction was followed by reverse phase HPLC and by assaying the residual thiol content of IFN-.beta..sub.ser17 by Ellman's assay. After 6.5 hours the reaction was terminated by adding 10% acetic acid to the reaction mixture to a pH of 5.5.

## Results

During the first 2-3 hours of the reaction, no oligomers or only low levels (<1%) of oligomers were formed. The level of oligomeric species decreased substantially during the later stages of the reaction. The oxidized product contained no free thiols and the desired oxidized product was obtained in yields exceeding 96%.

In comparison, an IFN-.beta..sub.ser17 oxidation was carried out in which o-iodosobenzoic acid (2 mg/ml) was added to the reaction mixture at once to a concentration of 5 mmol. This oxidation resulted in the formation of 10%-15% oligomers and only moderate recovery (80%) of the desired oxidized IFN was obtained.

## EXAMPLE 2

### Controlled Oxidation and Purification of IFN-.beta..sub.ser17

#### 1. Controlled Oxidation of Fully Reduced IFN-.beta..sub.ser17

The procedure described in Example 1 was used to prepare a solution of IFN-.beta..sub.ser17 in the fully reduced form except for the final pre-column step. A total of 1-2 mg/ml of the solution in DTT was run on a S-200 column and eluted with a sodium acetate buffer (50 mM, pH 5.5, 0.1% SDS). The S-200 IFN-.beta. pool was diluted to 0.1 mg/ml (5 micromolar IFN-.beta.) by adding sodium phosphate buffer, pH 7.5, 0.1% SDS. The pH of the solution was adjusted to 7.5. The oxidizing reagent iodosobenzoic acid (2 mg/ml) was added to the IFN-.beta. solution in order to obtain a final concentration of 40 micromolar. This IFN-oxidant solution was kept for 3 hours at room temperature under air and was gently stirred. The oxidation was followed by monitoring the thiol content of the protein solution using 2,2'-dithiodipyridine. The IFN-.beta. was concentrated to 5-10 mg/ml by using an Amicon cell and then run through a G-75 column using the same buffer that was used for the S-200 column. The final IFN-.beta. concentration was 2-3 mg/ml.

#### 2. Gel Filtration of the G-75 IFN-.beta. Pool by a G-25 Sephadex Column

A 2.6.times.70 cm glass column (Pharmacia) equipped with a packing reservoir was packed with 600 ml of pre-swelled gel solution of Sephadex G-25 (fine grade).

A total of 10 ml (1.44 mg/ml) of IFN-.beta. from the previous step was introduced to the column and eluted by using a 1 mM NaOH solution at pH 10.8. A flow rate of 250 ml/hr. was employed.

Approximately 98% of the protein peak was pooled together and analyzed for protein concentration, SDS and biological activity. In addition, reverse phase HPLC and SDS-PAGE gels were obtained.

#### 3. Reverse Phase HPLC Method

The protein sample that was collected from the column was acidified to pH 2-3 by adding concentrated tri-fluoro acetic acid (TFA). The HPLC traces were obtained by injecting 20-200 microliters to an Aquapore column. The elution of the sample was followed at 214 nm and performed by using a two-

solvent system and a gradient of 45-60% solvent B where solvent B is 0.1% TFA in acetonitrile and solvent A is 0.1% TFA in water. A flow rate of 2 ml/min. and a chart speed of 0.5 cm/min. were used. An Hewlett-Packard integrator was used to obtain the areas of the peaks.

#### 4. SDS Determinations

The SDS determinations were done by the acridine orange assay by placing an IFN-.beta. sample (0.5 ml) in a disposable 13.times.100 mm screw cap test tube followed by NaHSO.sub.4 (0.1 ml, 1.75M), acridine orange (0.1 ml, 1% wt/v) and finally toluene (1.5 ml). The test tubes were sealed and then vortexed for 2-3 minutes. The tubes were centrifuged for 5-10 minutes. After phase separation the organic layer was transferred to a quartz cuvette and the absorbance was measured at 500 nm versus a blank (0.5 ml of water).

#### 5. Formulation of IFN-.beta. with Normal Serum Albumin (NSA)

The formulation of IFN-.beta. with NSA was done by first calculating the final volume factor (F.V.) to obtain the NSA, dextrose and water volumes which were needed for the formulation.

The Final Volume=IFN-.beta./0.25

The NSA Volume=1.25/25.times.F.V.

The Dextrose Volume=1.25/50.times.F.V.

The H.sub.2 O Volume=F.V.-[V(IFN-.beta.)+V(HSA)+V(Dext)+V(neut)]

By a typical procedure (NSA, 3.9 ml, 25% solution obtained from Travenol) was mixed with 46 ml water. The pH was raised to 12 by employing a NaOH (2.5N) solution and monitoring it using an electrode. The IFN-.beta. solution (20 ml) was added and the mixture was held at pH 12 for 15 minutes. The pH was slowly lowered to 7.23 by using a HCl (2.5N or 0.25N) solution. The pH adjustment required about 10-15 minutes. Water (2 ml) and dextrose (1.9 ml, 50%) were added. The final IFN-.beta. concentration was 0.25 mg/ml. The final formulated solution was filtered through 0.2 microns Nalge sterilized filter and the filtered solution was used to fill the vials (1 ml in each vial). The vials were lyophilized and then capped. Upon lowering the pH to 7.3 the solution did not produce a haze and remained clear.

#### 6. Biological Activities

Biological activities were determined by using the yield reduction assay, which is the direct measurement of virus yields from IFN-treated cells. The assay protocol followed that of Stewart and Lockhart described in Journal of Virology, 6, 795-799 (1970).

#### 7. Results

The SDS-PAGE gel and the reverse phase HPLC trace indicate that the oxidized IFN-.beta. preparation was homogeneous and basically pure. Two runs on the oxidized material, summarized in Table I, indicate that the gel filtration G-25 column was effective in desalting the pool of oxidized IFN-.beta. to acceptable levels of SDS as determined by the acridine orange assay described by Anal. Biochem., Vol. 118, p. 138-141 (1981) with minor modifications of volumes and pH levels. The total time in the column can be as short as 30 minutes without affecting the efficiency of SDS removal. The DTT levels in the G-25 pool was hardly detectable. The recovery of IFN-.beta. from the column was essentially quantitative

and the dilution factor was less than two. Reverse phase HPLC traces indicate that the incubation of the oxidized form of IFN-.beta. in 1 mM NaOH at pH 10.8 did not introduce heterogeneity in the IFN-.beta. preparation, at least as determined by the HPLC method. The HPLC traces also indicate that the NSA-formulated and filtered IFN-.beta. HPLC trace did not change even after five days.

The SDS-PAGE reducing gel of the G-25 desalted protein also indicates that a single protein population was obtained.

The biological activity of the protein remained essentially the same during all the stages of the modified process, indicating that the oxidized IFN-.beta. remained essentially unchanged during the gel filtration stage.

TABLE I		Parameters and Assay Results		Run #1	Run #2
		Buffer	1 mM NaOH	1 mM NaOH	pH 10.8
of IFN-.beta. solution (ml)	10	10	IFN-.beta. concentration (mg/ml)	1	1.44
30 IFN-.beta. concentration after	0.36	0.48	desalting (mg/ml)	Recovery (%)	99
250 Total time in the column (min.)	32	37	SDS level (microgram/mg)	21	21
9.1 .times. 10.sup.7	9.2 .times. 10.sup.7				

### EXAMPLE 3

#### Oxidation of Fully Reduced IL-2

#### Preparation of Fully Reduced IL-2

IL-2 was recovered from E. coli K-12 strain MM294 that had been transformed with the plasmid pLW1 (deposited at the American Type Culture Collection on Aug. 4, 1983 under ATCC Number 39,405) as follows.

The genetically engineered E. coli were grown in a fermenter using the following growth medium.

(NH.sub.4).sub.2 SO.sub.4 72 mM KH.sub.2 PO.sub.4 21.6 mM Na.sub.3 Citrate 1.5 mM ZnSO.sub.4.7H.sub.2 O 60 mM MnSO.sub.4.H.sub.2 O 60 mM CuSO.sub.4.5H.sub.2 O 2 mM pH adjusted to 6.50 with 2.5 N NaOH autoclaved Sterile Additions (post autoclave) MgSO.sub.4.7H.sub.2 O 3 mM FeSO.sub.4 100 .mu.M L-tryptophan 70 mg/l Thiamine-HCl 20 mg/l Glucose 5 g/l Tetracycline 5 mg/l Ethanol (optional) 2% Casamino acid 2%

Dow Corning Antifoam B, 20% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

The pH of the fermenter was maintained at 6.8 with 5N KOH. Residual glucose was maintained between 5-10 g/l, dissolved oxygen at 40%, and temperature at 37.degree.+-1.degree. C. The casamino acids (20% stock solution) were added when the OD.sub.680 was about 10-15. Harvest was made two hours after ethanol addition. Three hours after adding the casamino concentrated solution, ethanol (95%) was added to get a final 2% concentration.

Cells were concentrated in a cross-flow ultrafiltration unit. The cells were washed and then disrupted in a Manton-Gaulin homogenizer. The cell disruptate was centrifuged. The paste was resuspended in 4M urea and let stand for 15-30 minutes. The urea washed fragments were centrifuged and resuspended in Tris-HCl buffer, pH 8.0. Solid SDS was added to a level of 5% SDS in order to solubilize the fragments.



The urea washed solution (200 ml) was reduced by DTT (10 mM) in the presence of EDTA (2.5 mM) at pH 8.0 and 60.degree. C. for 30 minutes. The suspension was centrifuged at 35K for two hours. The supernatant (35 ml) was loaded on a S-200 (K-50) column and eluted with buffer E (acetate pH 5.5, DTT (2 mM), EDTA (1 mM) and SDS (0.1%)) at a rate of 1.5 ml/min. The S-200 pool (270 ml, A.sub.280=1.77) was about 33% pure as determined by HPLC.

A portion of the S-200 pool (35 ml) was acidified with trifluoroacetic acid (TFA) to pH 2.0, and then loaded at 2.5 ml/min. on a semi-preparative (1.3 cm) C-4 Vydac column that was freshly prepared. This was done three times with 35 ml each loading. The solvent used for this semi-preparative purification was acetonitrile (0.1% TFA, buffer B) and the gradient that was used was 0% to 45% buffer B in 15 minutes followed by 45% to 75% of B in 200 minutes. The IL-2 pool came out as 76 ml (three runs) with an A.sub.280=0.326 that corresponds to about 25 mg of IL-2 and which is about 15% yield. This HPLC run was diluted into 1600 ml of Na.sub.2 PO.sub.4 buffer (0.1M, pH 7.0, 0.1% SDS) and then concentrated to 50 ml by using an Amicon cell equipped with a 76 mm PM- 10 membrane. The concentrate was washed with three volumes of 50 ml each of Na.sub.2 PO.sub.4 (50 mM) pH 7 buffer which contained 0.1% SDS. The final volume was 43 ml with an A.sub.280=0.65.

#### Controlled Oxidation of IL-2

Before the controlled oxidation was carried out, the total thiol content of the protein solution was determined with 2,2'-dithiodipyridine. This determination was necessary in order to calculate the minimum theoretical amount of o-iodosobenzoic acid that had to be added to the IL-2 solution to achieve complete oxidation. o-Iodosobenzoic acid solution (1 mM, 50 ml) was prepared by dissolving the compound (13.4 mg) in about 45 ml of H.sub.2 O by sonicating the mixture for a few minutes and then by stirring the slowly adding NaOH (1N) to dissolve the acid. The alkaline solution was added to obtain a final pH of 8.0 to 8.5. The volume of the oxidant solution was adjusted to a final volume of 50 ml. A sulfhydryl group determination was done in order to determine the total amount of oxidant needed for a complete oxidation. This corresponded to the total thiol concentration divided by two plus a 15 micromolar excess of the oxidant. The controlled oxidation was performed by adding the o-iodosobenzoic acid solution at a flow rate of 0.5 ml/hr. to the IL-2 solution (50 mM Na.sub.2 PO.sub.4, pH 7 or 7.5). The degree of the oxidation was monitored by reverse phase HPLC. The oxidation was stopped by lowering the pH of the solution to 5.5 using concentrated acetic acid. HPLC analysis of the oxidized product showed that it comprised about 80% of the desired oxidized IL-2, about 13% undesired isomers (the isomers were collected, assayed for IL-2 activity and found to be inactive) and about 6% reduced (unoxidized) IL-2.

A similar oxidation carried out at pH 7.5 provided significantly reduced conversion (54%) to the desired product.

#### Purification of Oxidized IL-2

The oxidized product was purified essentially by the same method that was described for the purification of reduced IL-2. Two loadings (20 ml each) were performed on the 1.3 cm column. The pooling of this HPLC run was determined by analyzing the individual fractions on an analytical reverse phase HPLC column. The total volume of the two HPLC runs corresponds to 18 ml with an A.sub.280=0.266, which is about 4.8 mg of oxidized and HPLC purified IL-2.

The organic solvent was removed by using a Speed-Vac. After completely drying the test tube from the organic solvent, sodium phosphate buffer (0.1M, pH 7.0) was added, followed by 0.1 ml SDS (1%) and sonication to ensure complete solubility. The total volume (3 ml) was loaded on a G-25 (medium)

column (1.5.times.23 cm) and eluted with a sodium phosphate buffer (2 mM, pH 7.5) with a flow rate of 45 ml/hr. The final volume that was obtained was 5.1 ml and 0.6 mg of IL-2 per ml. The protein concentration was determined by the Lowry method. An assay of the total content of alkyl sulphates was performed by the acridine orange method. The total alkyl sulphate residual content was about 42 micrograms per mg of IL-2.

Storage stability tests of this purified oxidized IL-2 at pH 7.5 at 5.degree. C. and room temperature indicated the material is stable (i.e., IL-2 activity remains unchanged) over prolonged time periods.

#### EXAMPLE 4

##### Oxidation of Fully-Reduced Des-AlaIL-2.sub.ser125

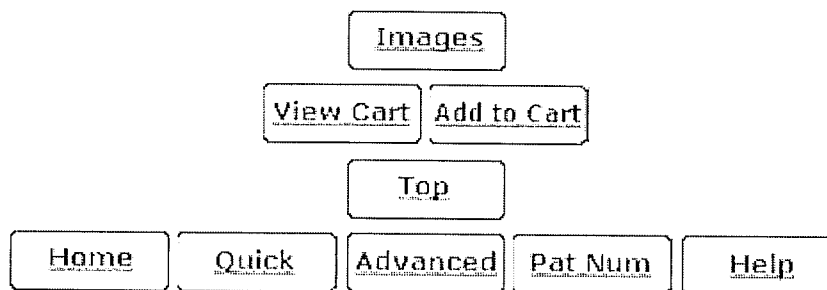
Des-ala IL-2.sub.ser125 is an IL-2 whose amino acid sequence differs from native human IL-2 by the absence of the initial N-terminal alanine residue and a serine substituted for cysteine at position 125. The strain of des-ala IL-2.sub.ser125 -producing E. coli used for this example was deposited in the ATCC on Mar. 6, 1984 under accession number 39,626.

These genetically engineered des-ala IL-2.sub.ser125 -producing E. coli were grown, the cells disrupted, and the cellular debris was recovered from the disruptate using the general procedures of Example 2. The cellular debris was extracted with 4M urea as in Example 2. The resulting paste was resuspended in aqueous buffer and solubilized with SDS. DTT, 150 mM, was added to the solution and the IL-2 was reduced by heating to 40.degree. C. at pH 8.5. The mixture was cooled and its pH adjusted to 5.0. The solution was then extracted with 2-butanol (1:1 v/v ratio) containing 1 mM DTT at room temperature. The organic extract was chromatographed on a S-200 column (as in Example 2) and then on a G-25 column using buffer E.

The G-25 pool was oxidized using the general procedure of Example 2. Following the oxidation, the oxidized product was purified by RP-HPLC using a Vydac TP214 packing and a solvent system of propanol in 1M acetic acid (gradient 35%-60% propanol over 200 min.). The recovered IL-2 was then diluted in 50 mM acetate buffer, pH 5.5, 6 mM EDTA, 0.1% SDS, and SDS was removed by G-25 column gel filtration using a 2 mM sodium phosphate, pH 7.5 buffer. The resulting oxidized, purified product is suitable for formulation for parenteral administration. The formulated composition may be lyophilized for storage.

Modification of the above-described modes for carrying out the invention that are obvious to those of skill in biochemical engineering are intended to be within the scope of the following claims.

\* \* \* \* \*



# EXHIBIT K

## PROLEUKIN<sup>®</sup> LABEL



Your Search Terms: PATENT BAYER

Version 1 - Published Jan 05, 2010

**PROLEUKIN - aldesleukin injection**  
Novartis Pharmaceuticals Corporation

-----  
**Proleukin**

**PROLEUKIN®**

**Aldesleukin For Injection**

**Rx Only**

#### **WARNINGS**

Therapy with PROLEUKIN® (aldesleukin) for injection should be restricted to patients with normal cardiac and pulmonary functions as defined by thallium stress testing and formal pulmonary function testing. Extreme caution should be used in patients with a normal thallium stress test and a normal pulmonary function test who have a history of cardiac or pulmonary disease.

PROLEUKIN should be administered in a hospital setting under the supervision of a qualified physician experienced in the use of anticancer agents. An intensive care facility and specialists skilled in cardiopulmonary or intensive care medicine must be available.

PROLEUKIN administration has been associated with capillary leak syndrome (CLS) which is characterized by a loss of vascular tone and extravasation of plasma proteins and fluid into the extravascular space. CLS results in hypotension and reduced organ perfusion which may be severe and can result in death. CLS may be associated with cardiac arrhythmias (supraventricular and ventricular), angina, myocardial infarction, respiratory insufficiency requiring intubation, gastrointestinal bleeding or infarction, renal insufficiency, edema, and mental status changes.

PROLEUKIN treatment is associated with impaired neutrophil function (reduced chemotaxis) and with an increased risk of disseminated infection, including sepsis and bacterial endocarditis. Consequently, preexisting bacterial infections should be adequately treated prior to initiation of PROLEUKIN therapy. Patients with indwelling central lines are particularly at risk for infection with gram positive microorganisms. Antibiotic prophylaxis with oxacillin, nafcillin, ciprofloxacin, or vancomycin has been associated with a reduced incidence of staphylococcal infections.

PROLEUKIN administration should be withheld in patients developing moderate to severe lethargy or somnolence; continued administration may result in coma.

#### **DESCRIPTION**

PROLEUKIN® (aldesleukin) for injection, a human recombinant interleukin-2 product, is a highly purified protein with a molecular weight of approximately 15,300 daltons. The chemical name is des-alanyl-1, serine-125 human interleukin-2. PROLEUKIN, a lymphokine, is produced by recombinant DNA technology using a genetically engineered *E. coli* strain containing an analog of the human interleukin-2 gene. Genetic engineering techniques were used to modify the human IL-2 gene, and the resulting expression clone encodes

a modified human interleukin-2. This recombinant form differs from native interleukin-2 in the following ways: a) PROLEUKIN is not glycosylated because it is derived from *E. coli*; b) the molecule has no N-terminal alanine; the codon for this amino acid was deleted during the genetic engineering procedure; c) the molecule has serine substituted for cysteine at amino acid position 125; this was accomplished by site specific manipulation during the genetic engineering procedure; and d) the aggregation state of PROLEUKIN is likely to be different from that of native interleukin-2.

The *in vitro* biological activities of the native nonrecombinant molecule have been reproduced with PROLEUKIN.<sup>1,2</sup>

PROLEUKIN is supplied as a sterile, white to off-white, lyophilized cake in single-use vials intended for intravenous (IV) administration. When reconstituted with 1.2 mL Sterile Water for Injection, USP, each mL contains 18 million IU (1.1 mg) PROLEUKIN, 50 mg mannitol, and 0.18 mg sodium dodecyl sulfate, buffered with approximately 0.17 mg monobasic and 0.89 mg dibasic sodium phosphate to a pH of 7.5 (range 7.2 to 7.8). The manufacturing process for PROLEUKIN involves fermentation in a defined medium containing tetracycline hydrochloride. The presence of the antibiotic is not detectable in the final product. PROLEUKIN contains no preservatives in the final product.

PROLEUKIN biological potency is determined by a lymphocyte proliferation bioassay and is expressed in International Units (IU) as established by the World Health Organization 1st International Standard for Interleukin-2 (human). The relationship between potency and protein mass is as follows:

18 million ( $18 \times 10^6$ ) IU PROLEUKIN = 1.1 mg protein

## CLINICAL PHARMACOLOGY

PROLEUKIN® (aldesleukin) has been shown to possess the biological activities of human native interleukin-2.<sup>1,2</sup> *In vitro* studies performed on human cell lines demonstrate the immunoregulatory properties of PROLEUKIN, including: a) enhancement of lymphocyte mitogenesis and stimulation of long-term growth of human interleukin-2 dependent cell lines; b) enhancement of lymphocyte cytotoxicity; c) induction of killer cell (lymphokine-activated (LAK) and natural (NK)) activity; and d) induction of interferon-gamma production.

The *in vivo* administration of PROLEUKIN in animals and humans produces multiple immunological effects in a dose dependent manner. These effects include activation of cellular immunity with profound lymphocytosis, eosinophilia, and thrombocytopenia, and the production of cytokines including tumor necrosis factor, IL-1 and gamma interferon.<sup>3</sup> *In vivo* experiments in murine tumor models have shown inhibition of tumor growth.<sup>4</sup> The exact mechanism by which PROLEUKIN mediates its antitumor activity in animals and humans is unknown.

### Pharmacokinetics

PROLEUKIN exists as biologically active, non-covalently bound microaggregates with an average size of 27 recombinant interleukin-2 molecules. The solubilizing agent, sodium dodecyl sulfate, may have an effect on the kinetic properties of this product.

The pharmacokinetic profile of PROLEUKIN is characterized by high plasma concentrations following a short IV infusion, rapid distribution into the extravascular space and elimination from the body by metabolism in the kidneys with little or no bioactive protein excreted in the urine. Studies of IV PROLEUKIN in sheep and humans indicate that upon completion of infusion, approximately 30% of the administered dose is detectable in plasma. This finding is consistent with studies in rats using radiolabeled PROLEUKIN, which demonstrate a rapid (<1 min) uptake of the majority of the label into the lungs, liver, kidney, and spleen.

The serum half-life ( $T_{1/2}$ ) curves of PROLEUKIN remaining in the plasma are derived from studies done in 52 cancer patients following a 5-minute IV infusion. These patients were shown to have a distribution and elimination  $T_{1/2}$  of 13 and 85 minutes, respectively.

Following the initial rapid organ distribution, the primary route of clearance of circulating PROLEUKIN is the kidney. In humans and animals, PROLEUKIN is cleared from the circulation by both glomerular filtration and peritubular extraction in the kidney.<sup>5-8</sup> This dual mechanism for delivery of PROLEUKIN to the proximal tubule may account for the preservation of clearance in patients with rising serum creatinine values. Greater than 80% of the amount of PROLEUKIN distributed to plasma, cleared from the circulation and presented to the kidney is metabolized to amino acids in the cells lining the proximal convoluted tubules. In humans, the mean clearance rate in cancer patients is 268 mL/min.

The relatively rapid clearance of PROLEUKIN has led to dosage schedules characterized by frequent, short infusions. Observed serum levels are proportional to the dose of PROLEUKIN.

### Immunogenicity

Fifty-seven of 77 (74%) metastatic renal cell carcinoma patients treated with an every 8-hour PROLEUKIN regimen and 33 of 50 (66%) metastatic melanoma patients treated with a variety of IV regimens developed low titers of non-neutralizing anti-PROLEUKIN antibodies. Neutralizing antibodies were not detected in this group of patients, but have been detected in 1/106 (<1%) patients treated with IV PROLEUKIN using a wide variety of schedules and doses. The clinical significance of anti-PROLEUKIN antibodies is unknown.

### Clinical Experience

Two hundred fifty-five patients with metastatic renal cell cancer (metastatic RCC) were treated with single agent PROLEUKIN in 7 clinical studies conducted at 21 institutions. Two hundred seventy patients with metastatic melanoma were treated with single agent PROLEUKIN in 8 clinical studies conducted at 22 institutions. Patients enrolled in trials of single agent PROLEUKIN were required to have an Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) of 0 or 1 and normal organ function as determined by cardiac stress test, pulmonary function tests, and creatinine  $\leq 1.5$  mg/dL. Patients with brain metastases, active infections, organ allografts and diseases requiring steroid treatment were excluded.

PROLEUKIN was given by 15 min IV infusion every 8 hours for up to 5 days (maximum of 14 doses). No treatment was given on days 6 to 14 and then dosing was repeated for up to 5 days on days 15 to 19 (maximum of 14 doses). These 2 cycles constituted 1 course of therapy. Patients could receive a maximum of 28 doses during a course of therapy. In practice >90% of patients had doses withheld. Metastatic RCC patients received a median of 20 of 28 scheduled doses of PROLEUKIN. Metastatic melanoma patients received a median of 18 of 28 scheduled doses of PROLEUKIN during the first course of therapy. Doses were withheld for specific toxicities (see “**DOSAGE AND ADMINISTRATION**” section, “**Dose Modifications**” subsection and “**ADVERSE REACTIONS**” section).

In the renal cell cancer studies (n=255), objective response was seen in 37 (15%) patients, with 17 (7%) complete and 20 (8%) partial responders (see Table I). The 95% confidence interval for objective response was 11% to 20%. Onset of tumor regression was observed as early as 4 weeks after completion of the first course of treatment, and in some cases, tumor regression continued for up to 12 months after the start of treatment. Responses were observed in both lung and non-lung sites (e.g., liver, lymph node, renal bed occurrences, soft tissue). Responses were also observed in patients with individual bulky lesions and high tumor burden.

In the metastatic melanoma studies (n=270), objective response was seen in 43 (16%) patients, with 17 (6%) complete and 26 (10%) partial responders (see Table I). The 95% confidence interval for objective response was 12% to 21%. Responses in metastatic melanoma patients were observed in both visceral and non-visceral sites (e.g., lung, liver, lymph node, soft tissue, adrenal, subcutaneous). Responses were also observed in patients with individual bulky lesions and large cumulative tumor burden.

**TABLE I PROLEUKIN CLINICAL RESPONSE DATA**

	Number of Responding Patients (response rate)	Median Response Duration in Months (range)
<b>Metastatic RCC</b>		
CR's	17 (7%)	80+* (7 to 131+)
PR's	20 (8%)	20 (3 to 126+)
PR's + CR's	37 (15%)	54 (3 to 131+)
<b>Metastatic Melanoma</b>		
CR's	17 (6%)	59+* (3 to 122+)
PR's	26 (10%)	6 (1 to 111+)
PR's + CR's	43 (16%)	9 (1 to 122+)

(+) sign means ongoing

\* Median duration not yet observed; a conservative value is presented which represents the minimum median duration of response.

An analysis of prognostic factors showed that a better ECOG performance status (see Table II) was significantly associated with response.

**TABLE II PROLEUKIN CLINICAL RESPONSE BY ECOG PERFORMANCE STATUS (PS)**

Pretreatment	METASTATIC RCC		METASTATIC MELANOMA	
ECOG PS	CR	PR	CR	PR
0	14/166 (8%)	16/166 (10%)	14/191 (7%)	22/191 (12%)

≥1	3/89 (3%)	4/89 (4%)	3/79 (4%)	4/79 (5%)
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## INDICATIONS AND USAGE

PROLEUKIN® (aldesleukin) is indicated for the treatment of adults with metastatic renal cell carcinoma (metastatic RCC).

PROLEUKIN is indicated for the treatment of adults with metastatic melanoma.

Careful patient selection is mandatory prior to the administration of PROLEUKIN. See “**CONTRAINDICATIONS**”, “**WARNINGS**” and “**PRECAUTIONS**” sections regarding patient screening, including recommended cardiac and pulmonary function tests and laboratory tests.

Evaluation of clinical studies to date reveals that patients with more favorable ECOG performance status (ECOG PS 0) at treatment initiation respond better to PROLEUKIN, with a higher response rate and lower toxicity (see “**CLINICAL PHARMACOLOGY**” section, “**Clinical Experience**” subsection and “**ADVERSE REACTIONS**” section). Therefore, selection of patients for treatment should include assessment of performance status.

Experience in patients with ECOG PS >1 is extremely limited.

## CONTRAINDICATIONS

PROLEUKIN® (aldesleukin) is contraindicated in patients with a known history of hypersensitivity to interleukin-2 or any component of the PROLEUKIN formulation.

PROLEUKIN is contraindicated in patients with an abnormal thallium stress test or abnormal pulmonary function tests and those with organ allografts. Retreatment with PROLEUKIN is contraindicated in patients who have experienced the following drug-related toxicities while receiving an earlier course of therapy:

- Sustained ventricular tachycardia (≥5 beats)
- Cardiac arrhythmias not controlled or unresponsive to management
- Chest pain with ECG changes, consistent with angina or myocardial infarction
- Cardiac tamponade
- Intubation for >72 hours
- Renal failure requiring dialysis >72 hours
- Coma or toxic psychosis lasting >48 hours
- Repetitive or difficult to control seizures
- Bowel ischemia/perforation
- GI bleeding requiring surgery

## WARNINGS

See boxed “**WARNINGS**”

Because of the severe adverse events which generally accompany PROLEUKIN® (aldesleukin) therapy at the recommended dosages, thorough clinical evaluation should be performed to identify patients with significant cardiac, pulmonary, renal, hepatic, or CNS impairment in whom PROLEUKIN is contraindicated. Patients with normal cardiovascular, pulmonary, hepatic, and CNS function may experience serious, life threatening or fatal adverse events. Adverse events are frequent, often serious, and sometimes fatal.

Should adverse events, which require dose modification occur, dosage should be withheld rather than reduced (see “**DOSAGE AND ADMINISTRATION**” section, “**Dose Modifications**” subsection).

PROLEUKIN has been associated with exacerbation of pre-existing or initial presentation of autoimmune disease and inflammatory disorders. Exacerbation of Crohn’s disease, scleroderma, thyroiditis, inflammatory arthritis, diabetes mellitus, oculo-bulbar myasthenia gravis, crescentic IgA glomerulonephritis, cholecystitis, cerebral vasculitis, Stevens-Johnson syndrome and bullous pemphigoid, has been reported following treatment with IL-2.

All patients should have thorough evaluation and treatment of CNS metastases and have a negative scan prior to receiving PROLEUKIN therapy. New neurologic signs, symptoms, and anatomic lesions following PROLEUKIN therapy have been reported in patients without evidence of CNS metastases. Clinical



manifestations included changes in mental status, speech difficulties, cortical blindness, limb or gait ataxia, hallucinations, agitation, obtundation, and coma. Radiological findings included multiple and, less commonly, single cortical lesions on MRI and evidence of demyelination. Neurologic signs and symptoms associated with PROLEUKIN therapy usually improve after discontinuation of PROLEUKIN therapy; however, there are reports of permanent neurologic defects. One case of possible cerebral vasculitis, responsive to dexamethasone, has been reported. In patients with known seizure disorders, extreme caution should be exercised as PROLEUKIN may cause seizures.

## PRECAUTIONS

### General

Patients should have normal cardiac, pulmonary, hepatic, and CNS function at the start of therapy (see “**PRECAUTIONS**” section, “**Laboratory Tests**” subsection). Capillary leak syndrome (CLS) begins immediately after PROLEUKIN® (aldesleukin) treatment starts and is marked by increased capillary permeability to protein and fluids and reduced vascular tone. In most patients, this results in a concomitant drop in mean arterial blood pressure within 2 to 12 hours after the start of treatment. With continued therapy, clinically significant hypotension (defined as systolic blood pressure below 90 mm Hg or a 20 mm Hg drop from baseline systolic pressure) and hypoperfusion will occur. In addition, extravasation of protein and fluids into the extravascular space will lead to the formation of edema and creation of new effusions.

Medical management of CLS begins with careful monitoring of the patient’s fluid and organ perfusion status. This is achieved by frequent determination of blood pressure and pulse, and by monitoring organ function, which includes assessment of mental status and urine output. Hypovolemia is assessed by catheterization and central pressure monitoring.

Flexibility in fluid and pressor management is essential for maintaining organ perfusion and blood pressure. Consequently, extreme caution should be used in treating patients with fixed requirements for large volumes of fluid (e.g., patients with hypercalcemia). Administration of IV fluids, either colloids or crystalloids is recommended for treatment of hypovolemia. Correction of hypovolemia may require large volumes of IV fluids but caution is required because unrestrained fluid administration may exacerbate problems associated with edema formation or effusions. With extravascular fluid accumulation, edema is common and ascites, pleural or pericardial effusions may develop. Management of these events depends on a careful balancing of the effects of fluid shifts so that neither the consequences of hypovolemia (e.g., impaired organ perfusion) nor the consequences of fluid accumulations (e.g., pulmonary edema) exceed the patient’s tolerance.

Clinical experience has shown that early administration of dopamine (1 to 5 µg/kg/min) to patients manifesting capillary leak syndrome, before the onset of hypotension, can help to maintain organ perfusion particularly to the kidney and thus preserve urine output. Weight and urine output should be carefully monitored. If organ perfusion and blood pressure are not sustained by dopamine therapy, clinical investigators have increased the dose of dopamine to 6 to 10 µg/kg/min or have added phenylephrine hydrochloride (1 to 5 µg/kg/min) to low dose dopamine (see “**ADVERSE REACTIONS**” section). Prolonged use of pressors, either in combination or as individual agents, at relatively high doses, may be associated with cardiac rhythm disturbances. If there has been excessive weight gain or edema formation, particularly if associated with shortness of breath from pulmonary congestion, use of diuretics, once blood pressure has normalized, has been shown to hasten recovery. **NOTE: Prior to the use of any product mentioned, the physician should refer to the package insert for the respective product.**

PROLEUKIN® (aldesleukin) treatment should be withheld for failure to maintain organ perfusion as demonstrated by altered mental status, reduced urine output, a fall in the systolic blood pressure below 90 mm Hg or onset of cardiac arrhythmias (see “**DOSAGE AND ADMINISTRATION**” section, “**Dose Modifications**” subsection). Recovery from CLS begins soon after cessation of PROLEUKIN therapy. Usually, within a few hours, the blood pressure rises, organ perfusion is restored and reabsorption of extravasated fluid and protein begins.

Kidney and liver function are impaired during PROLEUKIN treatment. Use of concomitant nephrotoxic or hepatotoxic medications may further increase toxicity to the kidney or liver.

Mental status changes including irritability, confusion, or depression which occur while receiving PROLEUKIN may be indicators of bacteremia or early bacterial sepsis, hypoperfusion, occult CNS malignancy, or direct PROLEUKIN-induced CNS toxicity. Alterations in mental status due solely to PROLEUKIN therapy may progress for several days before recovery begins. Rarely, patients have sustained permanent neurologic deficits (see “**PRECAUTIONS**” section, “**Drug Interactions**” subsection).

Exacerbation of pre-existing autoimmune disease or initial presentation of autoimmune and inflammatory disorders has been reported following PROLEUKIN alone or in combination with interferon (see “**PRECAUTIONS**” section, “**Drug Interactions**” subsection and “**ADVERSE REACTIONS**” section). Hypothyroidism, sometimes preceded by hyperthyroidism, has been reported following PROLEUKIN

treatment. Some of these patients required thyroid replacement therapy. Changes in thyroid function may be a manifestation of autoimmunity. Onset of symptomatic hyperglycemia and/or diabetes mellitus has been reported during PROLEUKIN therapy.

PROLEUKIN enhancement of cellular immune function may increase the risk of allograft rejection in transplant patients.

### Laboratory Tests

The following clinical evaluations are recommended for all patients, prior to beginning treatment and then daily during drug administration.

- Standard hematologic tests-including CBC, differential and platelet counts
- Blood chemistries-including electrolytes, renal and hepatic function tests
- Chest x-rays

Serum creatinine should be  $\leq 1.5$  mg/dL prior to initiation of PROLEUKIN treatment.

All patients should have baseline pulmonary function tests with arterial blood gases. Adequate pulmonary function should be documented ( $FEV_1 > 2$  liters or  $\geq 75\%$  of predicted for height and age) prior to initiating therapy.

All patients should be screened with a stress thallium study. Normal ejection fraction and unimpaired wall motion should be documented. If a thallium stress test suggests minor wall motion abnormalities further testing is suggested to exclude significant coronary artery disease.

Daily monitoring during therapy with PROLEUKIN should include vital signs (temperature, pulse, blood pressure, and respiration rate), weight, and fluid intake and output. In a patient with a decreased systolic blood pressure, especially less than 90 mm Hg, constant cardiac rhythm monitoring should be conducted. If an abnormal complex or rhythm is seen, an ECG should be performed. Vital signs in these hypotensive patients should be taken hourly.

During treatment, pulmonary function should be monitored on a regular basis by clinical examination, assessment of vital signs and pulse oximetry. Patients with dyspnea or clinical signs of respiratory impairment (tachypnea or rales) should be further assessed with arterial blood gas determination. These tests are to be repeated as often as clinically indicated.

Cardiac function should be assessed daily by clinical examination and assessment of vital signs. Patients with signs or symptoms of chest pain, murmurs, gallops, irregular rhythm or palpitations should be further assessed with an ECG examination and cardiac enzyme evaluation. Evidence of myocardial injury, including findings compatible with myocardial infarction or myocarditis, has been reported. Ventricular hypokinesia due to myocarditis may be persistent for several months. If there is evidence of cardiac ischemia or congestive heart failure, PROLEUKIN therapy should be held, and a repeat thallium study should be done.

### Drug Interactions

PROLEUKIN may affect central nervous function. Therefore, interactions could occur following concomitant administration of psychotropic drugs (e.g., narcotics, analgesics, antiemetics, sedatives, tranquilizers).

Concurrent administration of drugs possessing nephrotoxic (e.g., aminoglycosides, indomethacin), myelotoxic (e.g., cytotoxic chemotherapy), cardiotoxic (e.g., doxorubicin) or hepatotoxic (e.g., methotrexate, asparaginase) effects with PROLEUKIN may increase toxicity in these organ systems. The safety and efficacy of PROLEUKIN in combination with any antineoplastic agents have not been established.

In addition, reduced kidney and liver function secondary to PROLEUKIN treatment may delay elimination of concomitant medications and increase the risk of adverse events from those drugs.

Hypersensitivity reactions have been reported in patients receiving combination regimens containing sequential high dose PROLEUKIN and antineoplastic agents, specifically, dacarbazine, cis-platinum, tamoxifen and interferon-alfa. These reactions consisted of erythema, pruritus, and hypotension and occurred within hours of administration of chemotherapy. These events required medical intervention in some patients.

Myocardial injury, including myocardial infarction, myocarditis, ventricular hypokinesia, and severe rhabdomyolysis appear to be increased in patients receiving PROLEUKIN and interferon-alfa concurrently.

Exacerbation or the initial presentation of a number of autoimmune and inflammatory disorders has been observed following concurrent use of interferon-alfa and PROLEUKIN, including crescentic IgA glomerulonephritis, oculo-bulbar myasthenia gravis, inflammatory arthritis, thyroiditis, bullous pemphigoid, and Stevens-Johnson syndrome.

Although glucocorticoids have been shown to reduce PROLEUKIN-induced side effects including fever,

renal insufficiency, hyperbilirubinemia, confusion, and dyspnea, concomitant administration of these agents with PROLEUKIN may reduce the antitumor effectiveness of PROLEUKIN and thus should be avoided.<sup>12</sup>

Beta-blockers and other antihypertensives may potentiate the hypotension seen with PROLEUKIN.

#### **Delayed Adverse Reactions to Iodinated Contrast Media**

A review of the literature revealed that 12.6% (range 11-28%) of 501 patients treated with various interleukin-2 containing regimens who were subsequently administered radiographic iodinated contrast media experienced acute, atypical adverse reactions. The onset of symptoms usually occurred within hours (most commonly 1 to 4 hours) following the administration of contrast media. These reactions include fever, chills, nausea, vomiting, pruritus, rash, diarrhea, hypotension, edema, and oliguria. Some clinicians have noted that these reactions resemble the immediate side effects caused by interleukin-2 administration, however the cause of contrast reactions after interleukin-2 therapy is unknown. Most events were reported to occur when contrast media was given within 4 weeks after the last dose of interleukin-2. These events were also reported to occur when contrast media was given several months after interleukin-2 treatment.<sup>13</sup>

#### **Carcinogenesis, Mutagenesis, Impairment of Fertility**

There have been no studies conducted assessing the carcinogenic or mutagenic potential of PROLEUKIN.

There have been no studies conducted assessing the effect of PROLEUKIN on fertility. It is recommended that this drug not be administered to fertile persons of either gender not practicing effective contraception.

#### **Pregnancy**

##### *Pregnancy Category C.*

PROLEUKIN has been shown to have embryo-lethal effects in rats when given in doses at 27 to 36 times the human dose (scaled by body weight). Significant maternal toxicities were observed in pregnant rats administered PROLEUKIN by IV injection at doses 2.1 to 36 times higher than the human dose during critical period of organogenesis. No evidence of teratogenicity was observed other than that attributed to maternal toxicity. There are no adequate well-controlled studies of PROLEUKIN in pregnant women. PROLEUKIN should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

#### **Nursing Mothers**

It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from PROLEUKIN, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

#### **Pediatric Use**

Safety and effectiveness in children under 18 years of age have not been established.

#### **Geriatric Use**

There were a small number of patients aged 65 and over in clinical trials of PROLEUKIN; experience is limited to 27 patients, eight with metastatic melanoma and nineteen with metastatic renal cell carcinoma. The response rates were similar in patients 65 years and over as compared to those less than 65 years of age. The median number of courses and the median number of doses per course were similar between older and younger patients.

PROLEUKIN is known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. The pattern of organ system toxicity and the proportion of patients with severe toxicities by organ system were generally similar in patients 65 and older and younger patients. There was a trend, however, towards an increased incidence of severe urogenital toxicities and dyspnea in the older patients.

#### **ADVERSE REACTIONS**

The rate of drug-related deaths in the 255 metastatic RCC patients who received single-agent PROLEUKIN® (aldesleukin) was 4% (11/255); the rate of drug-related deaths in the 270 metastatic melanoma patients who received single-agent PROLEUKIN was 2% (6/270).

The following data on common adverse events (reported in greater than 10% of patients, any grade), presented by body system, decreasing frequency and by preferred term (COSTART) are based on 525 patients (255 with renal cell cancer and 270 with metastatic melanoma) treated with the recommended



infusion dosing regimen.

**TABLE III ADVERSE EVENTS OCCURRING IN  $\geq 10\%$  OF PATIENTS (n=525)**

Body System	% Patients	Body System	% Patients
<u>Body as a Whole</u>		<u>Metabolic and Nutritional Disorders</u>	
Chills	52	Bilirubinemia	40
Fever	29	Creatinine increase	33
Malaise	27	Peripheral edema	28
Asthenia	23	SGOT increase	23
Infection	13	Weight gain	16
Pain	12	Edema	15
Abdominal pain	11	Acidosis	12
Abdomen enlarged	10	Hypomagnesemia	12
<u>Cardiovascular</u>		Hypocalcemia	11
Hypotension	71	Alkaline phosphatase increase	10
Tachycardia	23	<u>Nervous</u>	
Vasodilation	13	Confusion	34
Supraventricular tachycardia	12	Somnolence	22
Cardiovascular disorder <sup>a</sup>	11	Anxiety	12
Arrhythmia	10	Dizziness	11
<u>Digestive</u>		<u>Respiratory</u>	
Diarrhea	67	Dyspnea	43
Vomiting	50	Lung disorder <sup>b</sup>	24
Nausea	35	Respiratory disorder <sup>c</sup>	11
Stomatitis	22	Cough increase	11
Anorexia	20	Rhinitis	10
Nausea and vomiting	19	<u>Skin and Appendages</u>	
<u>Hemic and Lymphatic</u>		Rash	42
Thrombocytopenia	37	Pruritus	24
Anemia	29	Exfoliative dermatitis	18
Leukopenia	16	<u>Urogenital</u>	
		Oliguria	63

<sup>a</sup> Cardiovascular disorder: fluctuations in blood pressure, asymptomatic ECG changes, CHF.

<sup>b</sup> Lung disorder: physical findings associated with pulmonary congestion, rales, rhonchi.

<sup>c</sup> Respiratory disorder: ARDS, CXR infiltrates, unspecified pulmonary changes.

The following data on life-threatening adverse events (reported in greater than 1% of patients, grade 4), presented by body system, and by preferred term (COSTART) are based on 525 patients (255 with renal cell cancer and 270 with metastatic melanoma) treated with the recommended infusion dosing regimen.

**TABLE IV LIFE-THREATENING (GRADE 4) ADVERSE EVENTS (n= 525)**

Body System	# (%) Patients	Body System	# (%) Patients
<u>Body as a Whole</u>		<u>Metabolic and Nutritional Disorders</u>	
Fever	5 (1%)	Bilirubinemia	13 (2%)
Infection	7 (1%)	Creatinine increase	5 (1%)
Sepsis	6 (1%)	SGOT increase	3 (1%)
<u>Cardiovascular</u>		Acidosis	4 (1%)
Hypotension	15 (3%)	<u>Nervous</u>	
Supraventricular tachycardia	3 (1%)	Confusion	5 (1%)

Cardiovascular disorder <sup>a</sup>	7 (1%)	Stupor	3 (1%)
Myocardial infarct	7 (1%)	Coma	8 (2%)
Ventricular tachycardia	5 (1%)	Psychosis	7 (1%)
Heart arrest	4 (1%)	<u>Respiratory</u>	
<u>Digestive</u>		Dyspnea	5 (1%)
Diarrhea	10 (2%)	Respiratory disorder <sup>c</sup>	14 (3%)
Vomiting	7 (1%)	Apnea	5 (1%)
<u>Hemic and Lymphatic</u>		<u>Urogenital</u>	
Thrombocytopenia	5 (1%)	Oliguria	33 (6%)
Coagulation disorder <sup>b</sup>	4 (1%)	Anuria	25 (5%)
		Acute kidney failure	3 (1%)

<sup>a</sup> Cardiovascular disorder: fluctuations in blood pressure.

<sup>b</sup> Coagulation disorder: intravascular coagulopathy.

<sup>c</sup> Respiratory disorder: ARDS, respiratory failure, intubation.

The following life-threatening (grade 4) events were reported by <1% of the 525 patients: hypothermia; shock; bradycardia; ventricular extrasystoles; myocardial ischemia; syncope; hemorrhage; atrial arrhythmia; phlebitis; AV block second degree; endocarditis; pericardial effusion; peripheral gangrene; thrombosis; coronary artery disorder; stomatitis; nausea and vomiting; liver function tests abnormal; gastrointestinal hemorrhage; hematemesis; bloody diarrhea; gastrointestinal disorder; intestinal perforation; pancreatitis; anemia; leukopenia; leukocytosis; hypocalcemia; alkaline phosphatase increase; BUN increase; hyperuricemia; NPN increase; respiratory acidosis; somnolence; agitation; neuropathy; paranoid reaction; convulsion; grand mal convulsion; delirium; asthma, lung edema; hyperventilation; hypoxia; hemoptysis; hypoventilation; pneumothorax; mydriasis; pupillary disorder; kidney function abnormal; kidney failure; acute tubular necrosis.

In an additional population of greater than 1,800 patients treated with PROLEUKIN-based regimens using a variety of doses and schedules (e.g., subcutaneous, continuous infusion, administration with LAK cells) the following serious adverse events were reported: duodenal ulceration; bowel necrosis; myocarditis; supraventricular tachycardia; permanent or transient blindness secondary to optic neuritis; transient ischemic attacks; meningitis; cerebral edema; pericarditis; allergic interstitial nephritis; tracheo-esophageal fistula.

In the same clinical population, the following fatal events each occurred with a frequency of <1%: malignant hyperthermia; cardiac arrest; myocardial infarction; pulmonary emboli; stroke; intestinal perforation; liver or renal failure; severe depression leading to suicide; pulmonary edema; respiratory arrest; respiratory failure. In patients with both metastatic RCC and metastatic melanoma, those with ECOG PS of 1 or higher had a higher treatment-related mortality and serious adverse events.

Most adverse reactions are self-limiting and, usually, but not invariably, reverse or improve within 2 or 3 days of discontinuation of therapy. Examples of adverse reactions with permanent sequelae include: myocardial infarction, bowel perforation/infarction, and gangrene.

In post-marketing experience, the following serious adverse events have been reported in a variety of treatment regimens that include interleukin-2: anaphylaxis; cellulitis; injection site necrosis; retroperitoneal hemorrhage; cardiomyopathy; cerebral hemorrhage; fatal endocarditis; hypertension; cholecystitis; colitis; gastritis; hepatitis; hepatosplenomegaly; intestinal obstruction; hyperthyroidism; neutropenia; myopathy; myositis; rhabdomyolysis; cerebral lesions; encephalopathy; extrapyramidal syndrome; insomnia; neuralgia; neuritis; neuropathy (demyelination); urticaria; pneumonia (bacterial, fungal, viral).

Exacerbation or initial presentation of a number of autoimmune and inflammatory disorders have been reported (see “**WARNINGS**” section, “**PRECAUTIONS**” section, “**Drug Interactions**” subsection). Persistent but nonprogressive vitiligo has been observed in malignant melanoma patients treated with interleukin-2. Synergistic, additive and novel toxicities have been reported with PROLEUKIN used in combination with other drugs. Novel toxicities include delayed adverse reactions to iodinated contrast media and hypersensitivity reactions to antineoplastic agents (see “**PRECAUTIONS**” section, “**Drug Interactions**” subsection).

Experience has shown the following concomitant medications to be useful in the management of patients on PROLEUKIN therapy: a) standard antipyretic therapy, including nonsteroidal anti-inflammatories (NSAIDs), started immediately prior to PROLEUKIN to reduce fever. Renal function should be monitored as some NSAIDs may cause synergistic nephrotoxicity; b) meperidine used to control the rigors associated with fever; c) H<sub>2</sub> antagonists given for prophylaxis of gastrointestinal irritation and bleeding; d) antiemetics and antidiarrheals used as needed to treat other gastrointestinal side effects. Generally these medications were discontinued 12 hours after the last dose of PROLEUKIN.

Patients with indwelling central lines have a higher risk of infection with gram positive organisms.<sup>9-11</sup> A reduced incidence of staphylococcal infections in PROLEUKIN studies has been associated with the use of antibiotic prophylaxis which includes the use of oxacillin, nafcillin, ciprofloxacin, or vancomycin. Hydroxyzine or diphenhydramine has been used to control symptoms from pruritic rashes and continued until resolution of pruritus. Topical creams and ointments should be applied as needed for skin manifestations. Preparations containing a steroid (e.g., hydrocortisone) should be avoided. **NOTE: Prior to the use of any product mentioned, the physician should refer to the package insert for the respective product.**

## OVERDOSAGE

Side effects following the use of PROLEUKIN® (aldesleukin) appear to be dose-related. Exceeding the recommended dose has been associated with a more rapid onset of expected dose-limiting toxicities. Symptoms which persist after cessation of PROLEUKIN should be monitored and treated supportively. Life-threatening toxicities may be ameliorated by the intravenous administration of dexamethasone, which may also result in loss of the therapeutic effects of PROLEUKIN.<sup>12</sup> **NOTE: Prior to the use of dexamethasone, the physician should refer to the package insert for this product.**

## DOSAGE AND ADMINISTRATION

The recommended PROLEUKIN® (aldesleukin) for injection treatment regimen is administered by a 15-minute IV infusion every 8 hours. Before initiating treatment, carefully review the “**INDICATIONS AND USAGE**”, “**CONTRAINDICATIONS**”, “**WARNINGS**”, “**PRECAUTIONS**”, and “**ADVERSE REACTIONS**” sections, particularly regarding patient selection, possible serious adverse events, patient monitoring and withholding dosage. The following schedule has been used to treat adult patients with metastatic renal cell carcinoma (metastatic RCC) or metastatic melanoma. Each course of treatment consists of two 5-day treatment cycles separated by a rest period.

600,000 IU/kg (0.037 mg/kg) dose administered every 8 hours by a 15-minute IV infusion for a maximum of 14 doses. Following 9 days of rest, the schedule is repeated for another 14 doses, for a maximum of 28 doses per course, as tolerated. During clinical trials, doses were frequently withheld for toxicity (see “**Clinical Experience**” and “**Dose Modifications**” subsections). Metastatic RCC patients treated with this schedule received a median of 20 of the 28 doses during the first course of therapy. Metastatic melanoma patients received a median of 18 doses during the first course of therapy.

## Retreatment

Patients should be evaluated for response approximately 4 weeks after completion of a course of therapy and again immediately prior to the scheduled start of the next treatment course. Additional courses of treatment should be given to patients only if there is some tumor shrinkage following the last course and retreatment is not contraindicated (see “**CONTRAINDICATIONS**” section). Each treatment course should be separated by a rest period of at least 7 weeks from the date of hospital discharge.

## Dose Modifications

Dose modification for toxicity should be accomplished by withholding or interrupting a dose rather than reducing the dose to be given. Decisions to stop, hold, or restart PROLEUKIN therapy must be made after a global assessment of the patient. With this in mind, the following guidelines should be used:

**Retreatment with PROLEUKIN is contraindicated in patients who have experienced the following toxicities:**

Body System	
Cardiovascular	Sustained ventricular tachycardia ( $\geq 5$ beats)
	Cardiac rhythm disturbances not controlled or unresponsive to management
	Chest pain with ECG changes, consistent with angina or myocardial infarction
	Cardiac tamponade
Respiratory	Intubation for >72 hours
Urogenital	Renal failure requiring dialysis >72 hours
Nervous	Coma or toxic psychosis lasting >48 hours
	Repetitive or difficult to control seizures
Digestive	Bowel ischemia/perforation
	GI bleeding requiring surgery

**Doses should be held and restarted according to the following:**

Body System	Hold dose for	Subsequent doses may be given if
Cardiovascular	Atrial fibrillation, supraventricular tachycardia or bradycardia that requires treatment or is recurrent or persistent	Patient is asymptomatic with full recovery to normal sinus rhythm
	Systolic bp <90 mm Hg with increasing requirements for pressors	Systolic bp $\geq$ 90 mm Hg and stable or improving requirements for pressors
	Any ECG change consistent with MI, ischemia or myocarditis with or without chest pain; suspicion of cardiac ischemia	Patient is asymptomatic, MI and myocarditis have been ruled out, clinical suspicion of angina is low; there is no evidence of ventricular hypokinesia
Respiratory	O <sub>2</sub> saturation <90%	O <sub>2</sub> saturation >90%
Nervous	Mental status changes, including moderate confusion or agitation	Mental status changes completely resolved
Body as a Whole	Sepsis syndrome, patient is clinically unstable	Sepsis syndrome has resolved, patient is clinically stable, infection is under treatment
Urogenital	Serum creatinine >4.5 mg/dL or a serum creatinine of $\geq$ 4 mg/dL in the presence of severe volume overload, acidosis, or hyperkalemia	Serum creatinine <4 mg/dL and fluid and electrolyte status is stable
	Persistent oliguria, urine output of <10 mL/hour for 16 to 24 hours with rising serum creatinine	Urine output >10 mL/hour with a decrease of serum creatinine >1.5 mg/dL or normalization of serum creatinine
Digestive	Signs of hepatic failure including encephalopathy, increasing ascites, liver pain, hypoglycemia	All signs of hepatic failure have resolved*
	Stool guaiac repeatedly >3-4+	Stool guaiac negative
Skin	Bullous dermatitis or marked worsening of pre-existing skin condition, avoid topical steroid therapy	Resolution of all signs of bullous dermatitis

\* Discontinue all further treatment for that course. A new course of treatment, if warranted, should be initiated no sooner than 7 weeks after cessation of adverse event and hospital discharge.

**Reconstitution and Dilution Directions: Reconstitution and dilution procedures other than those recommended may alter the delivery and/or pharmacology of PROLEUKIN and thus should be avoided.**

1. PROLEUKIN® (aldesleukin) is a sterile, white to off-white, preservative-free, lyophilized powder suitable for IV infusion upon reconstitution and dilution. **EACH VIAL CONTAINS 22 MILLION IU (1.3 MG) OF PROLEUKIN AND SHOULD BE RECONSTITUTED ASEPTICALLY WITH 1.2 ML OF STERILE WATER FOR INJECTION, USP. WHEN RECONSTITUTED AS DIRECTED, EACH ML CONTAINS 18 MILLION IU (1.1 MG) OF PROLEUKIN.** The resulting solution should be a clear, colorless to slightly yellow liquid. The vial is for single-use only and any unused portion should be discarded.
2. During reconstitution, the Sterile Water for Injection, USP should be directed at the side of the vial and the contents gently swirled to avoid excess foaming. **DO NOT SHAKE.**
3. The dose of PROLEUKIN, reconstituted with Sterile Water for Injection, USP (without preservative) should be diluted aseptically in 50 mL of 5% Dextrose Injection, USP (D5W) and infused over a 15-minute period.

In cases where the total dose of PROLEUKIN is 1.5 mg or less (e.g., a patient with a body weight of less than 40 kilograms), the dose of PROLEUKIN should be diluted in a smaller volume of D5W. Concentrations of PROLEUKIN below 30 µg/mL and above 70 µg/mL have shown increased variability in drug delivery. Dilution and delivery of PROLEUKIN outside of this concentration range should be avoided.

4. Glass bottles and plastic (polyvinyl chloride) bags have been used in clinical trials with comparable results. It is recommended that plastic bags be used as the dilution container since experimental studies suggest that use of plastic containers results in more consistent drug delivery. **In-line filters should not be used when administering PROLEUKIN.**

5. Before and after reconstitution and dilution, store in a refrigerator at 2° to 8°C (36° to 46°F). Do not



freeze. Administer PROLEUKIN within 48 hours of reconstitution. The solution should be brought to room temperature prior to infusion in the patient.

6. Reconstitution or dilution with Bacteriostatic Water for Injection, USP, or 0.9% Sodium Chloride Injection, USP should be avoided because of increased aggregation. PROLEUKIN should not be coadministered with other drugs in the same container.

7. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

## HOW SUPPLIED

PROLEUKIN® (aldesleukin) for injection is supplied in individually boxed single-use vials. Each vial contains  $22 \times 10^6$  IU of PROLEUKIN. Discard unused portion.

**NDC 0078-0495-61** Individually boxed single-use vial

Store vials of lyophilized PROLEUKIN in a refrigerator at 2° to 8°C (36° to 46°F). PROTECT FROM LIGHT. Store in carton until time of use.

Reconstituted or diluted PROLEUKIN is stable for up to 48 hours at refrigerated and room temperatures, 2° to 25°C (36° to 77°F). However, since this product contains no preservative, the reconstituted and diluted solutions should be stored in the refrigerator.

Do not use beyond the expiration date printed on the vial. **NOTE:** This product contains no preservative.

Rx Only

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**PRINCIPAL DISPLAY PANEL****Package Label – 22 million IU**

Rx Only NDC 0078-0495-61

Aldesleukin

Proleukin®

For Injection

22 million IU

1.3 mg

Single-Use Vial

For IV Use Only

Refrigerate

NDC 0078-0495-61

**Aldesleukin****Proleukin®**

For Injection

22 million IU 1.3 mg
-------------------------

SINGLE-USE VIAL  
FOR IV USE ONLY  
REFRIGERATE

Rx only

 **NOVARTIS**
**PROLEUKIN**

aldesleukin injection

**Product Information****Product Type**

HUMAN PRESCRIPTION DRUG

**NDC Product Code (Source)**

0078-0495

<b>Route of Administration</b>		INTRAVENOUS		<b>DEA Schedule</b>	
<b>Active Ingredient/Active Moiety</b>					
<b>Ingredient Name</b>		<b>Basis of Strength</b>		<b>Strength</b>	
ALDESLEUKIN (ALDESLEUKIN)		ALDESLEUKIN		1.1 mg in 1 mL	
<b>Inactive Ingredients</b>					
<b>Ingredient Name</b>		<b>Strength</b>			
MANNITOL		50 mg in 1 mL			
SODIUM PHOSPHATE, MONOBASIC		0.17 mg in 1 mL			
SODIUM LAURYL SULFATE		0.18 mg in 1 mL			
SODIUM PHOSPHATE, DIBASIC		0.89 mg in 1 mL			
WATER					
<b>Product Characteristics</b>					
<b>Color</b>		<b>Score</b>			
<b>Shape</b>		<b>Size</b>			
<b>Flavor</b>		<b>Imprint Code</b>			
<b>Contains</b>					
<b>Packaging</b>					
<b># NDC</b>		<b>Package Description</b>		<b>Multilevel Packaging</b>	
1 0078-0495-01		1 VIAL In 1 BOX		contains a VIAL, SINGLE-USE	
1		1 mL In 1 VIAL, SINGLE-USE		This package is contained within the BOX (0078-0495-01)	

**Marketing Information**

<b>Marketing Category</b>	<b>Application Number or Monograph Citation</b>	<b>Marketing Start Date</b>	<b>Marketing End Date</b>
BLA	BLA103293	05/06/1992	

**Labeler** - Novartis Pharmaceuticals Corporation (002147023)

Revised: 12/2009

Novartis Pharmaceuticals Corporation



EXHIBIT L  
PATENT 4,569,790

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( 1 of 1 )

**United States Patent****4,569,790****Koths , et al.****February 11, 1986**

Process for recovering microbially produced interleukin-2 and purified recombinant interleukin-2 compositions

**Abstract**

A process for recovering microbially produced IL-2 in a highly pure form from the cellular material of the microorganisms that produced it comprising: disrupting the cell membranes of the microorganisms; extracting the disruptate with a chaotropic agent, such as urea, that selectively extracts microbial proteins from the cellular material; solubilizing the IL-2 in the solid phase of the extraction mixture with an aqueous solution of a solubilizing agent, such as SDS, containing a reducing agent; and separating the IL-2 from the resulting solution by an optional extraction with 2-butanol or 2-methyl-2-butanol followed by gel filtration chromatography, oxidizing the IL-2 and purifying the oxidized IL-2 by RP-HPLC.

Inventors: **Koths; Kirston** (Berkeley, CA), **Thomson; James** (Albany, CA), **Kunitani; Michael** (Oakland, CA), **Wilson; Kenneth** (Walnut Creek, CA), **Hanisch; Wolf** (Oakland, CA)

Assignee: **Cetus Corporation** (Emeryville, CA)

Appl. No.: **06/594,223**

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14/435 (20060101); C07K 14/55 (20060101); C07K  
003/12 (); C12D 021/02 ()

**Field of Search:**

260/112R,112.5R 435/170,172,849,68

**References Cited [Referenced By]****U.S. Patent Documents**4315852

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4450103

May 1984

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*Primary Examiner:* Kight; John

*Assistant Examiner:* Draper; Garnette D.

*Attorney, Agent or Firm:* Halluin; Albert P. Ciotti; Thomas

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***Claims***

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We claim:

1. A process for recovering purified, oxidized human IL-2 from a transformed microorganism containing the IL-2 comprising:

(a) disrupting the cell membrane of the microorganism;

(b) extracting the disruptate with an aqueous solution of a chaotropic agent that extracts non IL-2 proteins selectively from the cellular material and from the IL-2;

(c) solubilizing the IL-2 in the solid phase of the extraction mixture with an aqueous solution of a solubilizing agent that forms a water soluble complex with the IL-2, said solution containing a reducing agent to thereby fully reduce the IL-2 in the complex;

(d) separating the reduced IL-2 from the resulting solution in the presence of the reducing agent;

(e) oxidizing the reduced IL-2;

(f) further purifying the resulting oxidized IL-2 product by gel filtration or reverse-phase high performance liquid chromatography as individual or combined steps; and

(g) recovering a purified oxidized recombinant human IL-2 composition having an IL-2 content of at least about 95% as determined by reducing SDS-PAGE analysis, an endotoxin content of less than about 0.1 nanograms/mg of IL-2 and substantially free of pyrogens as determined by the U.S.P. rabbit pyrogen test at a dosage of  $3.3 \times 10^5$  U/kg.

2. The process of claim 1 wherein the chaotropic agent is urea.

3. The process of claim 2 wherein the concentration of urea in the extraction mixture is in the range of about 3.5 M to 4.5 M.

4. The process of claim 1 wherein step (b) is carried out at a basic pH.

5. The process of claim 4 wherein the pH is in the range of about 8.1 to about 8.5.

6. The process of claim 1 wherein the solubilizing agent is sodium dodecyl sulfate or sodium lauryl sarcosine.

7. The process of claim 1 wherein step (f) is carried out by reverse phase high performance liquid chromatography at a pH in the range of 2.1 to 2.3 using a bonded phase wide pore silica gel and a gradient solvent system comprising an organic acid and an organic solvent for eluting IL-2.

8. The process of claim 7 wherein the solvent system is acetic acid-propanol, trifluoroacetic acid-propanol, or trifluoroacetic acid-acetonitrile.
  9. The process of claim 1 wherein step (f) is carried out by isolating an IL-2-containing fraction from the solution by gel filtration and purifying the resulting IL-2 from the fraction by reverse-phase high performance liquid chromatography.
  10. The process of claim 9 wherein the reverse phase high performance liquid chromatography is carried out at a pH in the range of 2.1 to 2.3 using a bonded phase wide pore silica gel and a gradient solvent system comprising an organic acid and an organic solvent for IL-2.
  11. The process of claim 10 wherein the solvent system is acetic acid-propanol, trifluoroacetic acid-propanol, or trifluoroacetic acid-acetonitrile.
  12. The process of claim 1 including the following additional steps of:
    - (i) extracting the IL-2 from the aqueous solution of (c) with 2-butanol or 2-methyl-2-butanol;
    - (ii) acid precipitating the IL-2 from the extract; and
    - (iii) purifying the acid precipitated IL-2 by gel filtration.
  13. The process of claim 12 wherein the chaotropic agent is urea, the concentration of urea in the extraction mixture is 3.5 M to 4.5 M, and the extraction of step (f) is carried out at a pH of 5 to 7.5.
  14. The process of claim 12 including purifying the resulting oxidized gel filtered product by reverse-phase high performance liquid chromatography.
  15. Purified recombinant human interleukin-2 (IL-2) composition wherein the IL-2 is unglycosylated and in oxidized form, having an IL-2 content of at least about 95% as determined by reducing SDS-PAGE analysis, an endotoxin content of less than about 0.1 nanogram/mg of IL-2 and substantially free of pyrogens as determined by the U.S.P. rabbit pyrogen test at a dosage of 3.3.times.10.sup.5 U/kg.
  16. The purified recombinant IL-2 composition of claim 15, wherein the IL-2 content is greater than about 98% as determined by reducing SDS-PAGE.
  17. The purified recombination human IL-2 composition of claim 15, wherein the IL-2 content is greater than about 98% as determined by RP-HPLC.
  18. The purified recombinant human IL-2 of claim 15, wherein the IL-2 is des-alanyl, serine.sub.125 IL-2.
  19. The purified recombinant human IL-2 of claim 16, wherein the IL-2 is des-alanyl, serine.sub.125 IL-2.
  20. The purified recombinant human IL-2 of claim 17 wherein the IL-2 is des-alanyl, serine.sub.125 IL-2.
  21. The process of claim 7 wherein the solvent system is acetic acid-propanol.
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### *Description*

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#### TECHNICAL FIELD

This invention is in the field of biochemical engineering. More particularly, the invention concerns a biochemical separation or recovery process in which interleukin-2 (IL-2) is separated or recovered from microorganisms that have been transformed to produce IL-2.

#### BACKGROUND ART

Native human IL-2 is an antigen-nonspecific, genetically unrestricted soluble factor produced by erythrocyte rosette positive T cells stimulated with antigens, mitogens and alloantigens. It is a protein with a reported molecular weight in the approximate range of 13,000 to 17,000 daltons (S. Gillis and J. Watson, J Exp Med (1980) 159:1709) and an isoelectric point in the approximate range of pH 6-8.5. Human IL-2 has a number of in vitro and in vivo effects including enhancing the proliferative responses of human peripheral blood mononuclear cells or murine thymocytes, enhancing the immune response in humans and in animals against bacterial, parasitic, fungal, protozoan and viral infections, and supporting the growth of continuous T cell lines.

IL-2 and IL-2 muteins in which the cysteine residue at amino acid 125 has been replaced with serine and/or the initial alanine has been eliminated have been produced microbially through genetic engineering techniques. Microbially produced IL-2 is not glycosylated and is produced in a reduced-state by the microorganisms. When purified and oxidized, these microbially produced IL-2s exhibit activity comparable to native human IL-2.

Procedures for purifying native IL-2 from T cells are described by Watson, J., et al, J Exp Med (1979) 150:849-861; Gillis, S., et al, J Immunology (1980) 124:1954-1962; Mochizuki, D. Y., et al, J Immun Meth (1980) 39:185-201; Welte, K., et al, J Exp Med (1982) 156:454-464; and European patent applications No. 83103582.9 (published 26 Oct. 1983 under No. 92163) and No. 83400938.3 (published 16 Nov. 1983 under No. 94317). In general these procedures involve precipitating proteins from culture supernatants with ammonium sulfate followed by a chromatographic fractionation.

Commonly owned copending U.S. patent application Ser. No. 353,360, filed 1 Mar. 1982 and Derynck, R., et al, Nature (1980) 287:193-197 describe procedures for recovering IFN-.beta. from IFN-.beta.-producing E. coli. The patent application describes a procedure in which IFN-.beta. is extracted from cellular material with 2-butanol or 2-methyl-2-butanol.

#### DISCLOSURE OF THE INVENTION

The invention is a process for recovering IL-2 from an IL-2-producing microorganism comprising:

- (a) disrupting the cell membrane of the microorganism;
- (b) extracting the disruptate with an aqueous solution of a chaotropic agent;
- (c) solubilizing the IL-2 in the solid phase of the extraction mixture with an aqueous solution of a solubilizing agent that forms a water soluble complex with the IL-2, said solution containing a reducing agent; and



(d) separating the IL-2 from the resulting solution in the presence of a reducing agent.

In preferred embodiments of this process the chaotropic agent is urea at a concentration of about 3.5 M to about 4.5 M in the extraction mixture, the solubilizing agent is sodium dodecyl sulfate (SDS) or sodium lauryl sarcosine (sarcosyl), the solubilized IL-2 is further extracted with 2-butanol or 2-methyl-2-butanol and the final separation is carried out by gel filtration, the resulting sized product is oxidized and the oxidized product is purified by reverse-phase high performance liquid chromatography (RP-HPLC).

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a flow diagram of two alternative embodiments of the invention process in which gel filtration chromatography is used as a final purification step. The embodiment designated Method 1A uses SDS as a solubilizing agent; the embodiment designated Method 1B uses sarcosyl as a solubilizing agent. The figure includes densitometer scans of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the product at various steps in the process.

FIG. 2 is an HPLC chromatogram and SDS-PAGE analysis of the product of Example 3.

FIG. 3 is an HPLC chromatogram of the product of Example 9.

FIG. 4 is a flow diagram of a preferred procedure for processing microbially produced IL-2.

#### MODES FOR CARRYING OUT THE INVENTION

As used herein the term "IL-2" denotes an unglycosylated protein that is (a) produced by a microorganism that has been transformed with a human interleukin-2 gene or a modification of the human interleukin-2 gene that encodes a protein having: (a) an amino acid sequence that is at least substantially identical to the amino acid sequence of native human interleukin-2 and (b) has biological activity that is common to native human interleukin-2. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not cause an adverse functional dissimilarity between the synthetic protein and native human interleukin-2. Examples of such proteins are the IL-2s described in European patent application No. 83101035.0 filed 3 Feb. 1983 (published 19 Oct. 1983 under publication No. 91539) and European patent application No. 82307036.2 filed 22 Dec. 1982 (published 14 Sept. 1983 under No. 88195), the IL-2s described in commonly owned U.S. Ser. No. 564,224, filed 20 Dec. 1983, now U.S. Pat. No. 4,518,584, issued May 21, 1985, which description is incorporated herein by reference, and the IL-2s described in the examples of this application.

As used herein the term "transformed microorganism" denotes a microorganism that has been genetically engineered to produce a protein that possesses native human interleukin-2 activity. Examples of transformed microorganisms are described in said European patent publications Nos. 88195 and 91539, said U.S. Pat. No. 4,518,584 and the examples of this application. Bacteria are preferred microorganisms for producing IL-2. Synthetic IL-2 may also be made by suitably transformed yeast and mammalian cells. *E. coli* is particularly preferred.

The transformed microorganisms are grown in a suitable growth medium, typically to an optical density (OD) of at least about 30 at 680 nm, and preferably between about 20 and 40 at 680 nm. The composition of the growth medium will depend upon the particular microorganism involved. The medium is an aqueous medium containing compounds that fulfill the nutritional requirements of the microorganism. Growth media will typically contain assimilable sources of carbon and nitrogen, energy



sources, magnesium, potassium and sodium ions, and optionally amino acids and purine and pyrimidine bases. (See Review of Medical Biology, Lange Medical Publications, 14th Ed pp 80-85 (1980).) In expression vectors involving the trp promoter, the tryptophane concentration in the medium is carefully controlled to become limiting at the time IL-2 expression is desired. Growth media for E. coli are well known in the art.

After the cells are harvested from the culture, they may be concentrated, if necessary, to about 20 to 150 mg/ml, preferably 80 to 100 mg/ml (OD 40 to 300, preferably 160 to 200 at 680 nm) by filtration, centrifugation, or other conventional methods.

Following concentration the cell membranes of the microorganisms are disrupted. The main purpose of disruption is to facilitate the following extraction and solubilization steps. Conventional cell disruption techniques such as homogenization, sonication, or pressure cycling may be used in this step of the process. Preferred methods are sonication or homogenization with a Manton-Gaulin homogenizer. The end point of the disruption step may be monitored by optical density, with the optical density of the suspension typically decreasing about 65% to 85%. In any event, the disruption should break substantially all of the cells so that substantially no intact cells are carried through to the solubilization step. Before the disruption, the pH of the liquid phase of the concentrate is adjusted, if necessary, to a level that facilitates removal of E. coli proteins in subsequent steps, while retaining IL-2 protein as an insoluble complex in the cellular debris. The pH may be so adjusted by adding suitable buffers. In most instances pHs in the range of about 8 to about 8.5 will be used.

The steps in the recovery process subsequent to the disruption step are primarily designed to separate the IL-2 from E. coli proteins to a high level of purity (preferably at least about 95% and more preferably at least about 98%) in good yields while maintaining the IL-2 in a reduced state. Simultaneously, these purification processes, in combination, also reduce pyrogenic substances in the final product to a level believed to be acceptable for parenteral administration to patients.

After the cells have been disrupted the particulate matter may be separated from the liquid phase of the disruptate and resuspended in an aqueous medium buffered to the optimal pH for the extraction. The particulate matter may optionally be washed with buffer at this stage to remove any water soluble E. coli proteins therein. In any event, the protein concentration of the cell suspension subjected to the extraction will usually be in the range of about 5 to about 60 mg/ml, preferably 20 to 40 mg/ml.

The extraction of E. coli proteins from the particulate cellular material may be carried out concurrently with the disruption or sequentially following the disruption. It is preferably carried out as a separate step following the disruption. The extractant is an aqueous solution of a chaotropic agent (i.e., a mild protein denaturant that dissociates hydrogen bonds and affects the tertiary structure of proteins). The extractant selectively removes the bulk of the E. coli proteins from the cellular debris leaving at least a substantial portion of the IL-2 associated (contained in or bound to) with the cellular debris. The selectivity is facilitated by the hydrophobicity of the IL-2 and the fact that it is in a reduced, insoluble state at a pH near the isoelectric point of the protein. In addition, a substantial portion of the IL-2 may be present in vivo as inclusion bodies of significant mass, as has been the case with other cloned proteins expressed at high levels in E. coli. Examples of extractants are urea and guanidinium hydrochloride (guanidinium hydrochloride should not be used when SDS is used as a solubilizing agent). Urea is preferred. The concentration of the chaotropic agent in the extraction mixture will depend upon the particular agent that is used and the amount of cellular material in the extraction mixture. In the case of urea, concentrations (final) between about 3.5 M and 4.5 M, preferably about 4 M, will be used in batch processes at 25.degree. C. If the extraction is run on a continuous basis over longer time periods it may be desirable to use lower concentrations. Temperatures in the range of 20.degree. C. to 25.degree. C. will normally be used in extraction, with room temperature being used for convenience. Mixing will typically be used

to enhance contact between the solution and particulate matter and thus decrease the time required to extract non-IL-2 proteins from the cellular debris. Kinetic analysis of the extraction process was performed on the supernatants using SDS-PAGE, and the extraction was found to be essentially complete by 15-30 min.

Following the extraction, the mixture is separated into solid and liquid phases. The IL-2 in the solid phase is then selectively solubilized by contacting the solid phase with a neutral, aqueous buffer containing a reducing agent and a solubilizing agent. Surface active agents (detergents) that have a suitable hydrophobic-hydrophilic balance to solubilize the hydrophobic IL-2 may be used. Alkali metal sulfates containing 10 to 14 carbon atoms and alkali metal alkyl sarcosinates are preferred solubilizing agents, with SDS and sarcosyl being particularly preferred.

The amount of solubilizing agent used in the solubilization will depend upon the particular agent. When SDS or sarcosyl are used, the preferred ratio (w/w) of SDS/sarcosyl to solid phase protein is about 0.5:1 to 1.4:1. The solubilizing medium also contains a sufficient amount of reducing agent to prevent the solubilized IL-2 from undergoing oxidation to any significant degree. Protein reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol may be used. The concentration of reducing agent such as DTT in the medium will usually range between about 5 to 20 mM. The solubilization will typically be carried out at temperatures in the range of 20.degree. C. to 25.degree. C. with mixing to facilitate contact between the solid phase and the solubilizing medium. Higher temperatures may solubilize unwanted E. coli proteins. The solubilization is considered complete when the sample has sat 15 min or the solution turns translucent. Insoluble material is separated after completing the solubilization.

After the IL-2 is solubilized the IL-2 may optionally be extracted from the aqueous solution under reducing conditions with 2-butanol or 2-methyl-2-butanol to remove additional E. coli proteins, notably including certain contaminants that have molecular weights very close to the IL-2. Conditions (e.g., ionic strengths in the range of 0.05 and 0.15) at which the aqueous solution and butanol are substantially immiscible are used. In carrying out the organic extraction the protein concentration of the aqueous solution is preferably adjusted, if necessary, to less than about 6 mg/ml, preferably about 0.5 to 4 mg/ml. Reducing conditions are maintained by carrying out the extraction in the presence of a reducing agent (e.g., DTT). The butanol will normally be added to the aqueous solution of solubilized IL-2 in volume ratios in the range of about 1:1 to about 3:1 (extractant:aqueous solution), preferably about 1:1. The extraction may be carried out in a batch or continuous operation. The temperature will normally be in the range of 20.degree. C. to 100.degree. C. and the pH will normally be about 4 to 9, preferably about 5 to 6. The time of contact between the solution and the butanol is not critical and relatively short times on the order of a few minutes may be used. After the extraction is complete, the aqueous phase and butanol phase are separated and the IL-2 is separated from the butanol phase. A preferred procedure for separating the IL-2 from the butanol phase is acid precipitation. This is done by adding the butanol phase to aqueous buffer, pH 7.5 until the organic phase is dissolved (approx. 2-3 vol buffer per vol of organic), and then lowering the pH to about 5.5 to 7.0, preferably 6.0 to 6.2, to cause the IL-2 to precipitate.

The next step in the process is to separate the IL-2 and any E. coli contaminants remaining after the extraction(s) and optimally from the solubilizing agent. Gel filtration chromatography, RP-HPLC, or a combination of gel filtration chromatography and RP-HPLC are used. The gel filtration chromatography is preferably carried out in two stages that remove both pyrogenic components and protein contaminants having molecular weights higher or lower than IL-2. (IL-2 has a molecular weight of about 15.5K daltons.) Gels that are capable of fractionating the solution to permit separation of the IL-2 from these contaminants are commercially available. Sephacryl S-200 is a preferred gel for removing the higher molecular weight components and Sephadex G-25, G-75 or G-100 gels are preferred for removing the low molecular weight contaminants. The gel filtrations will typically be run in buffered solutions (pH

5.5 to 7.0) containing about 0.1% to 1.0% solubilizing agent and about 1 to 10 mM reducing agent. The column will be sized to permit suitable resolution of the desired components.

RP-HPLC is an alternative to gel filtration. Also, RP-HPLC is capable of removing molecules from the solution that have molecular weights close to IL-2 and cannot, therefore, be removed completely by gel filtration. In addition, contaminants such as bacterial endotoxin are also removed effectively by RP-HPLC. Therefore, RP-HPLC may also be used as a final purification step after gel filtration. Supports (stationary phases) that provide good resolution of proteins may be used in the RP-HPLC. C-4, C-8, or C-18 on 300 angstrom pore-size supports are examples of preferred supports. The separation is carried out at an acidic pH of less than about 2.3, usually 2.1 to 2.3, in order to keep the IL-2 in solution. In this regard, the pH of the solution from the solubilization (gel filtration) will preferably be adjusted to this range. The solution is loaded into the RP-HPLC column and is adsorbed onto the stationary phase. A gradient solvent system comprising an organic acid such as acetic acid or trifluoroacetic acid and organic solvent such as propanol or acetonitrile is used to elute the IL-2 from the column. Acetic acid-propanol, trifluoroacetic acid-propanol, and trifluoroacetic acid-acetonitrile are preferred solvent systems. IL-2 elutes in the acetic acid-propanol system at about 40% propanol, in the trifluoroacetic acid-propanol system at about 50% propanol, and in the trifluoroacetic acid-acetonitrile system at about 62% acetonitrile. For convenience, the organic solvent content of the elutant will usually be increased rapidly to a level somewhat below the solvent concentration at which the IL-2 elutes followed by a slow gradient change in the range of about 0.1% to 1.0%/min.

As soon as the IL-2 is recovered from the chromatography step, it is lyophilized and resuspended in a neutral aqueous buffer containing the reducing agent (to keep the IL-2 in a reduced state) and the solubilizing agent (to keep it in solution). The IL-2 is stable in this form and may be stored for further treatment and formulation before being used.

An alternative and preferred procedure is to oxidize the IL-2 after it has been separated by gel filtration and purify the oxidized product by RP-HPLC or gel filtration followed by RP-HPLC. This results in efficient removal of contaminants surviving the gel filtration as well as unwanted oxidation products. A preferred oxidation procedure is described in a commonly owned U.S. patent application titled "Controlled Oxidation of Microbially Produced Cysteine-Containing Proteins", U.S. Ser. No. 594,351, filed Mar. 28, 1984, now abandoned in favor of U.S. Ser. No. 661,902, filed Oct. 17, 1984, U.S. Pat. No. 4,530,787. The relevant disclosure of that application is incorporated herein by reference. In said application Ser. No. 594,351 there is disclosed and claimed a preparative process for oxidizing fully reduced cysteine-containing microbially produced synthetic proteins, such as human IFN- $\beta$  or human IL-2, in a controlled manner so that the synthetic proteins have the same disulfide bridging as their native counterparts. The claimed process in said application includes oxidizing a fully reduced microbially produced synthetic protein having an amino acid sequence substantially identical to a useful protein which sequence includes cysteines which in the useful protein are linked intramolecularly to form a cystine in a controlled manner whereby said cysteines are oxidized selectively to form said cystine with minimal over-oxidation and formation of nonconforming cystine groups or oligomers comprising reacting the fully reduced microbially produced synthetic protein with o-iodosobenzoate in an aqueous medium at a pH at least about one-half pH unit below the pKa of said cysteines and wherein the concentration of synthetic protein in the reaction mixture is less than about 5 mg/ml and the mole ratio of o-iodosobenzoate to protein is at least stoichiometric, with the proviso that the o-iodosobenzoate is in excess in the terminal portion of the reaction. The process produces a novel cystine-containing protein, e.g., IL-2 preparation derived from synthetic microbially produced IL-2 having fully reduced cysteines comprising cystine-containing IL-2 which: (i) has the same disulfide bridging as native human IL-2; (ii) is substantially free of oligomers; and (iii) contains less than about 15% by weight of isomers, and preferably less than 1% by weight isomers, having disulfide bridging different from native human IL-2. RP-HPLC purification of the oxidized product may be carried out under the conditions described

above in the absence of a reducing agent and presence of a detergent at a concentration equal to or less than those used in the above described gel filtration.

The purity of the IL-2 after the chromatography step(s) is at least about 95% and usually at least about 98%. This highly pure material contains less than about 5 ng endotoxin, usually less than about 0.01 ng endotoxin per 100,000 Units IL-2 activity.

The invention process is further described by the following examples. These examples are not intended to limit the invention in any manner.

#### EXAMPLE 1

IL-2 was recovered from E. coli K-12 strain MM294 that had been transformed with the plasmid pLW1 (deposited at the American Type Culture Collection on 4 Aug. 1983 under accession number 39,405) as follows.

The E. coli were grown in a fermenter using the following growth medium.

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(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 150 mM KH<sub>2</sub>PO<sub>4</sub> 21.6 mM Na<sub>3</sub>Citrate 1.5 mM ZnSO<sub>4</sub>·7H<sub>2</sub>O 30 mM MnSO<sub>4</sub>·H<sub>2</sub>O 30 mM CuSO<sub>4</sub>·5H<sub>2</sub>O 1 mM pH adjusted to 6.50 with 2.5 N NaOH autoclaved Sterile Additions (post autoclave) MgSO<sub>4</sub>·7H<sub>2</sub>O 3 mM FeSO<sub>4</sub> 100 .mu.M L-tryptophan 14 mg/l Thiamine-HCl 20 mg/l Glucose 5 g/l Tetracycline 5 mg/l Ethanol (optional) 2% Casamino acids 2%

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Dow Corning Antifoam B, 20% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

The pH of the fermenter was maintained at 6.8 with 5N KOH. Residual glucose was maintained between 5-10 g/l, dissolved oxygen at 40%, and temperature at 37.degree.+-1.degree. C. The casamino acids (20% stock solution) were added when the OD<sub>680</sub> was about 10. Harvest was made three hr after the OD<sub>680</sub> reached about 20.

The harvested material was concentrated by hollow fiber filtration and/or centrifugation. Twenty to forty g (wet weight) of the concentrate were resuspended in 200 ml of 50 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.1-8.5) (Tris/EDTA buffer). The suspension was centrifuged at 3,000-4,000.times.g for 10 min, the supernatant was removed, and the solids were resuspended in 200 ml Tris/EDTA buffer at 4.degree. C. The suspension was loaded into a sonicator (Heat Systems, Model W-375) and sonicated at 4.degree. C. for 45 min (end point=OD<sub>680</sub> reduction of about 85%) using large probe, pulsing with 50% duty on power setting "9". An alternative disruption technique is to pass the suspension three times through a Manton-Gaulin homogenizer on M-1 setting. Cellular debris was separated from the disruptate by centrifuging at 4,500.times.g for 10 min.

The cellular debris was resuspended in 60 ml Tris/EDTA buffer at room temperature and an equal volume of 8 M urea (Schwarz/Mann ultrapure) in Tris/EDTA buffer was added to the suspension over five min with rapid stirring (final urea concentration, 4 M). After continued slow stirring for 15-30 min, the suspension was centrifuged at 12,000.times.g for 15 min to recover extracted cellular debris. (If a solid phase does not form, the supernatant is withdrawn, an equal volume of Tris/EDTA buffer is added and the mixture is recentrifuged.)

The extracted cellular debris is then resuspended in 9 ml of 50 mM sodium phosphate (pH 6.8), 1 mM EDTA, 10 mM DTT at 20.degree. C. One ml of 20% SDS is added to the suspension, and the



suspension is mixed vigorously for 5 min. The liquid phase is recovered from the suspension by centrifuging at 12,000.times.g for 10 min at room temperature. The liquid phase was then heated to 40.degree. C. for 15 min to insure that the IL-2 in the solution is fully reduced. A sample of this crude extract was analyzed by 15% SDS-PAGE. FIG. 1 shows a densitometer scan of that analysis (product of Method 1A) indicating the extract contained about 37% IL-2.

IL-2 was separated from the solution by gel filtration chromatography as follows. The solution was loaded onto a 2.6 cm.times.100 cm S-200 column run in 50 mM sodium phosphate (pH 6.8), 1 mM EDTA, 1 mM DTT, 1% SDS. The column effluent was collected in 4 ml fractions with samples of the fractions analyzed in 15% SDS-PAGE minigels stained with Coomassie blue. The fractions containing the fewest contaminants (minimizing contaminants at about 35K daltons, 16-18K daltons, and 12K daltons) were pooled and concentrated to 5-10 ml by ultrafiltration (Amicon YM5 ultrafilter). The concentrate was loaded onto a 2.6 cm.times.100 cm G-100 column, run as above except that the SDS concentration was 0.1% rather than 1%. Fractions were analyzed by SDS-PAGE and the purest fractions were pooled. The drawing shows a densitometer scan of the chromatographed product. Analysis indicated the product was 98% pure and contained 0.5 ng endotoxin/ 100,000 units of IL-2 activity as measured by the limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA). The N-terminal amino acid sequence of this IL-2 is the same as the native human molecule except that the initial N-terminal alanine is missing.

#### EXAMPLE 2

The procedure of Example 1 was repeated using 2% sarcosyl instead of 2% SDS as a solubilizing agent and using sarcosyl in place of SDS in the chromatography columns. FIG. 1 shows the densitometer scan for this crude extract using sarcosyl as a solubilizing agent (crude extract of Method 1B). As indicated, the use of sarcosyl in place of SDS gave improved purity (58% vs 37%) at similar IL-2 yield (50% vs 60%).

#### EXAMPLE 3

The procedure of Example 1 was repeated through the steps preceding urea extraction and was then solubilized and clarified as described.

The IL-2 was separated from the solution by RP-HPLC as follows. The solution was diluted 10-fold in 0.1% trifluoroacetic acid (TFA) and was applied to a 4.6 mm I.D..times.5 cm L. Brownlee Aquapore RP-300 column equilibrated in 0.1% TFA. The IL-2 was eluted with a gradient of 30%-60% acetonitrile containing 0.1% TFA over 45 min. The yield of IL-2 activity following HPLC was 80-100%. FIG. 2 shows a silver-stained SDS-PAGE analysis of this product.

#### EXAMPLE 4

The procedure of Example 1 was repeated through the steps preceding gel filtration chromatography. The soluble, clarified, reduced material was subjected to G-100 chromatography in 0.1% SDS as described in Example 1. The pooled peak fractions of IL-2 were further purified by RP-HPLC as described in Example 3. The resulting purified, reduced IL-2 was oxidized and subjected to RP-HPLC as described in Example 3.

#### EXAMPLE 5

The procedure of Example 1 was repeated through the steps preceding the G-100 column. The procedure for Example 3 was repeated using a solvent system of propanol in 1 M acetic acid. The IL-2 was eluted

with a gradient of 35%-60% propanol over 200 min. Column dimensions were either 10 mm ID.times.30 cm L or 48 mm ID.times.50 cm L, and the column was packed with a bonded phase wide-pore silica gel. The bonded phase wide-pore silica used was Vydac TP214. The purity and yield of product was comparable to that of Example 3.

#### EXAMPLE 6

The procedure of Example 3 was repeated using a solvent system of propanol in 0.1% TFA. The IL-2 was eluted with a gradient of 35%-60% propanol over 120 min. The column and support materials were the same as in Example 5. The purity and yield of product were comparable to that of Example 3.

#### EXAMPLE 7

The procedure of Example 1 was repeated except that the E. coli-produced IL-2 was one designated des-Ala Ser.sub.125 IL-2. The amino acid sequence of this IL-2 is different from that of the native molecule in that the cysteine at position 125 has been changed to serine and the initial N-terminal alanine residue is missing. The E. coli that produce this IL-2 were made by the techniques described in said U.S. Ser. No. 4,518,584. Strains of des-Ala Ser.sub.125 IL-2-producing E. coli were deposited in the American Type Culture Collection on Sept. 26, 1983 under accession number 39,452 and on Mar. 6, 1984 under accession number 39,626.

#### EXAMPLE 8

The procedure of Example 1 was repeated except that the IL-2 was recovered from E. coli K-12 strain that had been transformed with the plasmid pLW55 (deposited in the American Type Culture Collection on Nov. 18, 1983 under accession number 39,516). The amino acid sequence of this molecule is different from that of the native molecule in that it has an N-terminal methionine and the cysteine at position 125 has been changed to serine.

#### EXAMPLE 9

Des-Ala Ser.sub.125 IL-2-producing E. coli were grown, the cells disrupted and the cellular debris recovered from the disruptate using the general procedures of Example 1. The cellular debris was suspended in 50 mM Tris, 1 mM EDTA pH 8.5 buffer at a ratio of about 1:4.5 (w/v). DTT was added to a final concentration of 25 mM. 8 M urea in the same buffer was slowly added with stirring to a final concentration of 4 M and then allowed to mix at room temperature for 30 min. After 30 min, the insoluble material remaining was centrifuged. The resulting paste was resuspended in 50 mM sodium phosphate buffer, 1 mM EDTA pH 7.0. The suspension was then solubilized by addition of solid SDS to a final concentration of 5% w/v.

The 5% SDS solution was diluted to 2% SDS with 0.1 M Na.sub.2 PO.sub.4, pH 8.0. The protein concentration was determined, the pH was adjusted to 8.5, and DTT to 50 mM and EDTA to 2 mM were added. The mixture was heated to 40.degree. C. under N.sub.2 to reduce the IL-2. The mixture was then cooled and the pH was adjusted to 5.0.

The solution was then extracted at a 1:1 ratio (v/v) with 2-butanol containing 1 mM DTT at room temperature. Residence time was 2-2.5 min. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic extract was separated and its pH was adjusted to 8.0 with NaOH. The extract was then added slowly to 0.1% SDS in 10 mM Na.sub.2 PO.sub.4, 2 mM DTT, pH 6 and stirred for 15-20 min. The resulting precipitate was separated and the resulting paste was resuspended in 5% SDS in PBS. The solution was clarified by centrifugation and reduced as above.

Following reduction the solution was adjusted to pH 5.5 with acetic acid. The solution was purified by gel filtration using S-200 and G-25 columns. The resulting purified, reduced IL-2 was oxidized, and the oxidized product was purified by G-25 chromatography followed by RP-HPLC as in Example 3. The resulting purified recombinant IL-2 product has an IL-2 content greater than about 95% as determined by reducing SDS-PAGE analysis, an endotoxin content of less than about 0.1 nanograms/mg of IL-2, and it is substantially free of pyrogens as determined by the U.S.P. rabbit pyrogen test at a dosage of 3.3.times.10.sup.5 U/kg. As previously indicated, the endotoxin content is less than about 5 nanograms, and preferably less than 0.01 nanograms endotoxin per 100,000 units IL-2 activity. Typically, the purified recombinant IL-2 products purified by the process of the invention have an IL-2 content greater than 98% as determined by reducing SDS-PAGE or RP-HPLC, as shown in FIG. 3 in addition to being substantially free of endotoxins and pyrogens as indicated above.

A variation of the process described in Example 9, such as might be used to produce IL-2 on a larger scale, is shown in FIG. 4. The process shown in FIG. 4 differs from that described in Example 9 as regards (1) minor changes in the buffers, (2) use of an acetic acid-propanol (Example 5) solvent system in the RP-HPLC, and (3) the inclusion of post-oxidation dilution/diafiltration S-200 gel filtration, and ultrafiltration steps. The process as shown in FIG. 4 may be modified with various refinements, for example, following the second S-200 column pass, in 1% SDS, the IL-2 solution is diluted 1:10 to give a 0.1% SDS concentration and then diafiltered against 10 mM phosphate buffer at a pH of 7.5 and 5 ppm SDS. The solution is then concentrated as required for appropriate use dosage.

Modifications of the above described modes for carrying out the invention that are obvious to those of skill in the fields of biochemistry, biochemical engineering, and related arts are intended to be within the scope of the following claims.

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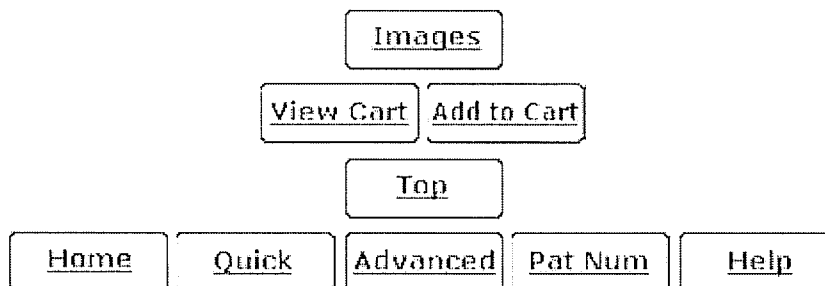




EXHIBIT M  
PATENT 4,604,377

USPTO PATENT FULL-TEXT AND IMAGE DATABASE

<a href="#">Home</a>	<a href="#">Quick</a>	<a href="#">Advanced</a>	<a href="#">Pat Num</a>	<a href="#">Help</a>
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<a href="#">View Cart</a>		<a href="#">Add to Cart</a>		
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( 1 of 1 )

United States Patent  
Fernandes , et al.

4,604,377  
August 5, 1986

Pharmaceutical compositions of microbially produced interleukin-2

**Abstract**

A sterile, stable lyophilized formulation of selectively oxidized microbially produced recombinant IL-2 in which the recombinant IL-2 is admixed with a water soluble carrier such as mannitol that provides bulk, and a sufficient amount of sodium dodecyl sulfate to ensure the solubility of the recombinant IL-2 in water. The formulation is suitable for reconstitution in aqueous injections for parenteral administration and it is stable and well tolerated in human patients. FIG. 1 illustrates a preferred method of purifying recombinant IL-2 suitable for use in preparing the formulations of the present invention.

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[\*] Notice: The portion of the term of this patent subsequent to July 23, 2002 has been disclaimed.

Appl. No.: **06/715,152**

Filed: **March 21, 1985**

**Related U.S. Patent Documents**

<u>Application Number</u>	<u>Filing Date</u>	<u>Patent Number</u>	<u>Issue Date</u>
594350	Mar., 1984		
<b>Current U.S. Class:</b>			
424/85.2 ; 424/278.1; 435/69.52; 435/948; 514/2; 530/351			
<b>Current International Class:</b>			
A61K 9/19 (20060101); C07K 14/435 (20060101); C07K 14/55 (20060101); A61K 47/20 (20060101); A61K 47/28 (20060101); A61K 47/26 (20060101); A61K 045/02 (); A61K 037/02 (); A61K 039/39 (); C07K 013/00 ()			
<b>Field of Search:</b>			
260/112R 435/68,70,172.3,948 424/85,88,87 935/11,12 514/2,8			

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*Primary Examiner:* Schain; Howard E.  
*Attorney, Agent or Firm:* Halluin; Albert P.

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***Parent Case Text***

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**CROSS REFERENCE TO RELATED APPLICATIONS**

This application is a continuation-in-part of U.S. Ser. No. 594,350, filed Mar. 28, 1984, now abandoned, the disclosure of which is incorporated herein by reference. This application is related to U.S. application Ser. Nos. 564,224, now U.S. Pat. No. 4,518,584 filed Dec. 20, 1983; 594,351, filed Mar. 28, 1984, now abandoned, 661,902 filed Oct. 17, 1984, now U.S. Pat. No. 4,530,787; 594,223, filed Mar. 28, 1984; 594,250, filed Mar. 28, 1984; the disclosures of which are incorporated herein by reference.

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***Claims***

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What is claimed is:

1. A recombinant IL-2 composition suitable for reconstituting in a pharmaceutically acceptable aqueous vehicle for parenteral administration to a patient to provide IL-2 therapy comprising a sterile lyophilized mixture of:

(a) a therapeutically effective amount of a selectively oxidized microbially produced recombinant IL-2 that is substantially free of non-IL-2 protein, and it is at least 95% pure recombinant IL-2, and contains less than about 5 ng endotoxin per 100,000 units of IL-2 activity;

(b) a physiologically acceptable water soluble carrier that does not affect the stability of the selectively oxidized microbially produced IL-2 adversely; and

(c) a sufficient amount of a surface active agent to ensure the water solubility of the selectively oxidized, microbially produced hydrophobic recombinant IL-2.

2. The composition of claim 1 wherein the oxidized microbially produced recombinant IL-2 includes

less than about 5% by weight non-IL-2 protein and the surface active agent is sodium dodecyl sulfate (SDS) or sodium deoxycholate.

3. The composition of claim 1 wherein the oxidized microbially produced IL-2 constitutes about 0.02% to 3.85% by weight of the mixture.

4. The composition of claim 1 wherein the recombinant IL-2 is des ala IL-2.sub.ser125.

5. The composition of claim 1 wherein the water soluble carrier is mannitol.

6. The composition of claim 2 wherein the sodium dodecyl sulfate is present at about 100 to about 250 .mu.g per mg of IL-2.

7. The composition of claim 2 wherein the recombinant IL-2 is des ala IL-2.sub.ser125, the recombinant IL-2 protein includes less than about 5% by weight non-IL-2 protein, the IL-2 constitutes about 0.015% to 3.85% by weight of the mixture, the water soluble carrier is mannitol, and the sodium dodecyl sulfate is present at about 100 to about 250 .mu.g per mg of IL-2.

8. A pharmaceutical composition for providing therapy to a patient comprising a sterile solution of:

(a) the mixture of claim 1 dissolved in

(b) a pharmaceutically acceptable aqueous parenteral injection, said solution containing in the range of about 0.01 mg to about 2 mg of the selectively oxidized microbially produced recombinant IL-2 ml.

9. A pharmaceutical composition for providing IL-2 therapy to a patient comprising a sterile solution of:

(a) the mixture of claim 2 dissolved in

(b) a pharmaceutically acceptable aqueous parenteral injection, said solution containing in the range of about 0.01 mg to about 2 mg of the selectively oxidized microbially produced recombinant IL-2 ml.

10. A pharmaceutical composition for providing IL-2 therapy to a patient comprising a sterile solution of:

(a) the mixture of claim 3 dissolved in

(b) a pharmaceutically acceptable aqueous parenteral injection, said solution containing in the range of about 0.01 mg to about 2 mg of the selectively oxidized microbially produced recombinant IL-2 per ml.

11. A pharmaceutical composition for providing IL-2 therapy to a patient comprising a sterile solution of:

(a) a mixture of claim 4 dissolved in

(b) a pharmaceutically acceptable aqueous parenteral injection, said solution containing in the range of about 0.01 mg to about 2 mg of the selectively oxidized microbially produced recombinant IL-2 per ml.

12. A pharmaceutical composition for providing IL-2 therapy to a patient comprising a sterile solution of:

(a) the mixture of claim 5 dissolved in

(b) a pharmaceutically acceptable aqueous parenteral injection, said solution containing in the range of about 0.01 to about 2 mg of the selectively oxidized microbially produced recombinant IL-2 per ml.

13. A pharmaceutical composition for providing IL-2 therapy to a patient comprising a sterile solution of:

(a) the mixture of claim 6 dissolved in

(b) a pharmaceutically acceptable aqueous parenteral injection, said solution containing in the range of about 0.01 mg to about 2 mg of the selectively oxidized microbially produced recombinant IL-2 per ml.

14. A pharmaceutical composition for providing therapy to a patient comprising a sterile solution of:

(a) the mixture of claim 7 dissolved in

(b) a pharmaceutically acceptable aqueous parenteral injection, said solution containing in the range of about 0.01 mg to about 2 mg of the selectively oxidized microbially produced recombinant IL-2 per ml.

15. The pharmaceutical composition of claim 8 wherein the aqueous parenteral vehicle is water for injection.

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### *Description*

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#### TECHNICAL FIELD

This invention is in the field of pharmaceuticals. More particularly, it relates to pharmaceutical formulations of microbially produced interleukin-2.

#### BACKGROUND ART

Interleukin-2, a lymphokine which is produced by normal peripheral blood lymphocytes and induces proliferation of antigen or mitogen stimulated T cells after exposure to plant lectins, antigens, or other stimuli, was first described by Morgan, D. A., et. al., Science (1976) 193:1007-1008. Then called T cell growth factor because of its ability to induce proliferation of stimulated T lymphocytes, it is now recognized that in addition to its growth factor properties it modulates a variety of functions of immune system cells in vitro and in vivo and has been renamed interleukin-2 (IL-2). IL-2 is one of several lymphocyte-produced messenger-regulatory molecules that mediate immunocyte interactions and functions.

IL-2 was initially made by cultivating human peripheral blood lymphocytes (PBL) or other IL-2-producing cell lines. See, for instance, U.S. Pat. No. 4,401,756. Recombinant DNA technology has provided an alternative to PBLs and cell lines for producing IL-2. Taniguchi, T., et al., Nature (1983) 302:305-310 and Devos, R., Nucleic Acids Research (1983) 11:4307-4323 have reported cloning the human IL-2 gene and expressing it in microorganisms.

Belgian Pat. No. 898,016, granted Nov. 14, 1983 and U.S. application Ser. No. 564,224 (U.S. Pat. No.

4,518,584 granted May 21, 1985) describe muteins of IL-2 in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been deleted or replaced with a neutral amino acid, such as serine. These muteins possess IL-2 biological activity. The Belgian patent states that the recombinant muteins may be formulated and administered as with native IL-2 by combining them with aqueous vehicles and injecting them intravenously, subcutaneously, or the like.

## DISCLOSURE OF THE INVENTION

One aspect of the present invention is an IL-2 composition suitable for reconstituting in a pharmaceutically acceptable aqueous vehicle for parenteral administration to a patient to provide IL-2 therapy comprising a sterile lyophilized mixture of:

- (a) a therapeutically effective amount of oxidized microbially produced recombinant IL-2 that is substantially free of non-IL-2 protein;
- (b) a pharmaceutically acceptable water soluble carrier that does not affect the stability of the microbially produced IL-2 adversely; and
- (c) a sufficient amount of surface active agent such as alkali metal sulfates, e.g., sodium dodecyl sulfate (SDS), alkali metal sarcosinates or sodium deoxycholate to ensure the water solubility of the microbially produced recombinant IL-2.

Preferably, the recombinant IL-2 has been selectively oxidized such that the cysteines at positions 58 and 105 form a disulfide bond to render the molecule biologically active.

Another aspect of this invention is a pharmaceutical composition for providing therapy to a patient comprising a sterile solution of:

- (a) the above described mixture dissolved in
- (b) a pharmaceutically acceptable aqueous parenteral vehicle, said solution containing in the range of about 0.01 mg to about 2 mg of the microbially produced recombinant IL-2 per ml.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow diagram of a preferred procedure for processing and purifying microbially produced recombinant IL-2.

## MODES FOR CARRYING OUT THE INVENTION

As used herein the term "IL-2" denotes an unglycosylated protein that is produced by a microorganism that has been transformed with a human interleukin-2 DNA sequence or a modification of the human interleukin-2 DNA sequence that encodes a protein having: (a) an amino acid sequence that is at least substantially identical to the amino acid sequence of native human interleukin-2 including the disulfide bond of the cysteines at positions 58 and 105, and (b) has biological activity that is common to native human interleukin-2. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not cause an adverse functional dissimilarity between the synthetic protein and native human interleukin-2. Examples of such proteins are the recombinant IL-2s described in European patent application No. 83101035.0 filed Feb. 3, 1983 (published Oct. 19, 1983 under publication No. 91539) and European patent application No. 82307036.2 filed Dec. 22, 1982 (published Sept. 14, 1983 under No. 88195 ), the



recombinant IL-2 muteins described in European patent application No. 83306221.9 filed Oct. 13, 1983 (published May 30, 1984 under No. 109748) which is the equivalent to Belgian Pat. No. 893,016, commonly owned U.S. Pat. No. 4,518,584, and the recombinant IL-2s described in this application.

As used herein the term "transformed microorganism" denotes a microorganism that has been genetically engineered to produce a protein that possesses native human interleukin-2 activity. Examples of transformed microorganisms are described in said European patent publications Nos. 88,198; 91,539, 109,748 and U.S. Ser. No. 564,224. Bacteria are preferred microorganisms for producing IL-2. A typical transformed microorganism useful in the present invention is *E. coli* K-12 strain MM294 transformed with plasmid pLW1 (deposited at the American Type Culture Collection on Aug. 4, 1983 by Cetus Corporation under the provisions of the Budapest Treaty and having accession No. 39,405). Synthetic recombinant IL-2 may also be made by suitably transformed yeast and mammalian cells. *E. coli* is particularly preferred host organism.

FIG. 1 illustrates a flow diagram for processing and purifying microbially-produced recombinant IL-2. The description that follows provides further details of this flow diagram. Reference is also made to U.S. Ser. Nos. 594,351; 661,902; 594,223 and 594,250; the disclosures of which are incorporated herein by reference.

The transformed microorganisms are grown in a suitable growth medium, typically to an optical density (OD) of at least about 30 to 680 nm, and preferably between about 20 and 40 at 680 nm. The composition of the growth medium will depend upon the particular microorganism involved. The medium is an aqueous medium containing compound that fulfill the nutritional requirements of the microorganism. Growth media will typically contain assimilable sources of carbon and nitrogen, energy sources, magnesium, potassium and sodium ions, and optionally amino acids and purine and pyrimidine bases. (See Review of Medical Biology, Lange Medical Publications, 14th Ed pp 80-95 (1980.)) In expression vectors involving the *trp* promoter, the tryptophane concentration in the medium is carefully controlled to become limiting at the time IL-2 expression is desired. Growth media for *E. coli* are well known in the art. A preferred growth method is described in U.S. Pat. No. 4,499,188, granted Feb. 12, 1985.

After the cells are harvested from the culture, they may be concentrated, if necessary, to about 20 to 150 mg/ml, preferably 80 to 100 mg/ml (OD 40 to 300, preferably 160 to 200 at 680 nm) by filtration, centrifugation, or other conventional methods.

Following concentration the cell membranes of the microorganisms are disrupted. The main purpose of disruption is to facilitate the following extraction and solubilization steps. Conventional cell disruption techniques such as homogenization, sonication, or pressure cycling may be used in this step of the process. Preferred methods are sonication or homogenization with a Manton-Gaulin homogenizer. The end point of the disruption step may be monitored by optical density, with the optical density of the suspension typically decreasing about 65% to 85%. In any event, the disruption should break substantially all of the cells so that substantially no intact cells are carried through to the solubilization step. Before the disruption, the pH of the liquid phase of the concentrate is adjusted, if necessary, to a level that facilitates removal of *E. coli* proteins in subsequent steps, while retaining recombinant IL-2 protein as an insoluble complex in the cellular debris. The pH may be so adjusted by adding suitable buffers. In most instances pHs in the range of about 8 to about 8.5 will be used.

The steps in the recovery process subsequent to the disruption step as shown in FIG. 1 are primarily designed to separate the IL-2 from *E. coli* proteins to a high level of purity (preferably at least about 95% and more preferably at least about 98%) in good yields while maintaining the IL-2 in a reduced state. Simultaneously, these purification processes, in combination, also reduce pyrogenic substances in



the final product to a level believed to be acceptable for parenteral administration to patients.

After the cells have been disrupted the particulate matter may be separated from the liquid phase of the disruptate and resuspended in an aqueous medium buffered to the optimal pH for the extraction. The particulate matter may optionally be washed with buffer at this stage to remove any water soluble *E. coli* proteins therein. In any event, the protein concentration of the cell suspension subjected to the extraction will usually be in the range of about 5 to about 60 mg/ml, preferably 20 to 40 mg/ml.

The extraction of *E. coli* proteins from the particulate cellular material may be carried out concurrently with the disruption or sequentially following the disruption. It is preferably carried out as a step following the disruption. The extractant is an aqueous solution of a chaotropic agent (i.e., a mild protein denaturant that dissociates hydrogen bonds and affects the tertiary structure of proteins). The extractant selectively removes the bulk of the *E. coli* proteins from the cellular debris leaving at least a substantial portion of the recombinant IL-2 associated (contained in or bound to) with the cellular debris. The selectivity is facilitated by the hydrophobicity of the recombinant IL-2 and the fact that it is in a reduced, insoluble state at a pH near the isoelectric point of the protein. In addition, a substantial portion of the recombinant IL-2 may be present in vivo as inclusion bodies of significant mass, as has been the case with other cloned proteins expressed at high levels in *E. coli*. Examples of extractants are urea and guanidinium hydrochloride (guanidinium hydrochloride should not be used when SDS is used as a solubilizing agent). Urea is preferred. The concentration of the chaotropic agent in the extraction mixture will depend upon the particular agent that is used and the amount of cellular material in the extraction mixture. In the case of urea, concentrations (final) between about 3.5 M and 4.5 M, preferably about 4 M, will be used in batch processes at 25.degree. C. If the extraction is run on a continuous basis over longer time periods it may be desirable to use lower concentrations. Temperatures in the range of 20.degree. C. to 25.degree. C. will normally be used in extraction, with room temperature being used for convenience. Mixing will typically be used to enhance contact between the solution and particulate matter and thus decrease the time required to extract non-IL-2 proteins from the cellular debris. Kinetic analysis of the extraction process was performed on the supernatants using SDS-PAGE, and the extraction was found to be essentially complete by 15-30 minutes.

Following the extraction, the mixture is separated into solid and liquid phases. The recombinant IL-2 in the solid phase is then selectively solubilized by contacting the solid phase with a neutral, aqueous buffer containing a reducing agent and a solubilizing agent. Physiologically acceptable surface active agents (detergents) that have a suitable hydrophobic-hydrophilic balance to solubilize the hydrophobic recombinant IL-2 may be used. Alkali metal sulfates containing 10 to 14 carbon atoms and alkali metal alkyl sarcosinates are preferred solubilizing agents, with SDS and sarcosyl being particularly preferred.

The amount of solubilizing agent used in the solubilization will depend upon the particular agent. When SDS or sarcosyl are used, the preferred ratio (w/w) of SDS/sarcosyl to solid phase protein is about 0.5:1 to 1.4:1. The solubilizing medium also contains a sufficient amount of reducing agent to prevent the solubilized IL-2 from undergoing oxidation to any significant degree. Protein reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol may be used. The concentration of reducing agent such as DTT in the medium will usually range between about 5 to 20 mM. The solubilization will typically be carried out at temperatures in the range of 20.degree. C. to 25.degree. C. with mixing to facilitate contact between the solid phase and the solubilizing medium. Higher temperatures may solubilize unwanted *E. coli* proteins. The solubilization is considered complete when the sample has sat 15 minutes or the solution turns translucent. Insoluble material is separated after completing the solubilization.

After the IL-2 is solubilized the IL-2 may optionally be extracted from the aqueous solution under reducing conditions with 2-butanol or 2-methyl-2-butanol to remove additional *E. coli* proteins, notably including certain contaminants that have molecular weights very close to the IL-2. Conditions (e.g.,

ionic strengths in the range of 0.05 and 0.15) at which the aqueous solution and butanol are substantially immiscible are used. In carrying out the organic extraction the protein concentration of the aqueous solution is preferably adjusted, if necessary, to less than about 6 mg/ml, preferably about 0.5 to 4 mg/ml. Reducing conditions are maintained by carrying out the extraction in the presence of a reducing agent (e.g., DTT). The butanol will normally be added to the aqueous solution of solubilized IL-2 in volume ratios in the range of about 1:1 to about 3:1 (extractant:aqueous solution), preferably about 1:1. The extraction may be carried out in a batch or continuous operation. The temperature will normally be in the range of 20.degree. C. to 100.degree. C. and the pH will normally be about 4 to 9, preferably about 5 to 6. The time of contact between the solution and the butanol is not critical and relatively short times on the order of a few minutes may be used. After the extraction is complete, the aqueous phase and butanol phase are separated and the IL-2 is separated from the butanol phase. A preferred procedure for separating the IL-2 from the butanol phase is acid precipitation. This is done by adding the butanol phase to aqueous buffer, pH 7.5 until the organic phase is dissolved (approx. 2-3 vol buffer per vol of organic), and then lowering the pH to about 5.5 to 7.0, preferably 6.0 to 6.2, to cause the IL-2 to precipitate.

The next step in the process is to separate the recombinant IL-2 and any E. coli contaminants remaining after the extraction(s) and optimally from the solubilizing agent. Gel filtration chromatography, RP-HPLC, or a combination of gel filtration chromatography and RP-HPLC are used. The gel filtration chromatographic is preferably carried out in two stages that remove pyrogenic components and protein contaminants having molecular weights higher or lower than recombinant IL-2. (Recombinant IL-2 has a molecular weight of about 15.5K daltons.) Gels that are capable of fractionating the solution to permit separation of the IL-2 from these contaminants are commercially available. Sephacryl S-200 is a preferred gel for removing the higher molecular weight components and Sephadex G-25, G-75 or G-100 gels are preferred for moving the low molecular weight contaminants. The gel filtrations will typically be run in buffered solutions (pH 5.5 to 7.0) containing about 0.1% to 1.0% solubilizing agent and about 1 to 10 nM reducing agent. The column will be sized to permit suitable resolution of the desired components.

RP-HPLC is an alternative to gel filtration. Also, RP-HPLC is capable of removing molecules from the solution that have molecular weights close to recombinant IL-2 and cannot, therefore, be removed completely by gel filtration. In addition, contaminants such as bacterial endotoxin are also removed effectively by RP-HPLC. Therefore, RP-HPLC may also be used as a final purification step after gel filtration. Supports (stationary phases) that provide good resolution of proteins may also be used as a final purification step after gel filtration. Supports (stationary phases) that provide good resolution of proteins may be used in the RP-HPLC. C-4, C-8, or C-18 on 300 angstrom pore-size supports are examples of preferred supports. The separation is carried out at an acidic pH of less than about 2.3, usually 2.1 to 2.3 in order to keep the IL-2 in solution. In this regard, the pH of the solution from the solubilization (gel filtration) will preferably be adjusted to this range. The solution is loaded into the RP-HPLC column and is absorbed onto the stationary phase. A gradient solvent system comprising an organic acid such as acetic acid or trifluoroacetic acid and organic solvent such as propanol or acetonitrile is used to elute the recombinant IL-2 from the column. Acetic acid-propanol, trifluoroacetic acid-propanol, and trifluoroacetic acid-acetonitrile are preferred solvent systems. Recombinant IL-2 elutes in the acetic acid-propanol system at about 40% propanol, in the trifluoroacetic acid-propanol system at about 50% propanol, and in the trifluoroacetic acid-acetonitrile system at about 62% acetonitrile. For convenience, the organic solvent content of the elutant will usually be increased rapidly to a level somewhat below the solvent concentration at which the recombinant IL-2 elutes followed by a slow gradient change in the range of about 0.1% to 1.0%/min.

As soon as the recombinant IL-2 is recovered from the chromatography step, it is lyophilized and resuspended in a neutral aqueous buffer containing the reducing agent (to keep the recombinant IL-2 in a

reduced state) and the solubilizing agent (to keep it in solution). The recombinant IL-2 is stable in this form and may be stored for further treatment and formulation before being used.

An alternative and preferred procedure is to selectively oxidize, under controlled conditions, the recombinant IL-2 after it has been separated by gel filtration and purify the oxidized product by RP-HPLC or gel filtration followed by RP-HPLC. This results in efficient removal of contaminants surviving the gel filtration as well as unwanted oxidation products. A preferred oxidation procedure is to selectively oxidize a fully reduced microbially produced synthetic recombinant IL-2 protein having an amino acid sequence substantially identical to the recombinant IL-2 protein which sequence includes cysteines which in the useful protein are linked intramolecularly at positions 58 to 105 to form a cystine in a controlled manner so that the cysteines are oxidized selectively to form the cystine at positions 58 and 105. The efficiency of the controlled and selective oxidation is improved if a recombinant IL-2 mutein is used such as described and claimed in Belgian Pat. No. 898,016 and U.S. Pat. No. 4,518,584. In such case the cysteine at position 125 is deleted or replaced with a neutral amino acid thus preventing incorrect intramolecular bonds and/or intermolecular bonds with the cysteine at position 125 during oxidation which may also form dimers or polymers of IL-2. In this process the fully reduced microbially produced synthetic recombinant IL-2 protein is preferably reacted with o-iodosobenzoate, which oxidizes cysteines selectively in an aqueous medium, at a pH at least about one-half pH unit below the pK<sub>a</sub> of said cysteines, wherein the concentration of synthetic protein in the reaction mixture is less than about 5 mg/ml and the mol ratio of o-iodosobenzoate to protein is at least stoichiometric, with the proviso that the o-iodosobenzoate is in excess in the terminal portion of the reaction. This selective oxidation produces a biologically active molecule. RP-HPLC purification of the selectively oxidized product may be carried out under the conditions described above in the absence of a reducing agent and presence of a detergent at a concentration equal to or less than those used in the above described gel filtration.

The purity of the recombinant IL-2 after the chromatography step(s) is at least about 95% and usually at least about 98%. This highly pure material contains less than about 5 ng endotoxin, usually less than about 0.01 ng endotoxin per 100,000 Units IL-2 activity.

The formulation of recombinant IL-2 in accordance with this invention may be carried out as a separate operation using purified, selectively oxidized IL-2 or in an operation that is integrated with the purification of the selectively oxidized IL-2. In the latter case, the starting material for the formulation is a recombinant IL-2-containing product from a reverse phase high performance liquid chromatography (RP-HPLC) treatment of the selectively oxidized product, preferably recombinant IL-2 selectively oxidized by the RP-HPLC product (pool) will comprise a solution of recombinant IL-2 in a water-organic solvent mixture. The nature of the organic solvent will depend upon the solvent system used in RP-HPLC. Examples of systems that may be used are combinations of an organic acid such as acetic acid or trifluoroacetic acid and organic solvent such as propanol or acetonitrile.

The first step in formulating the recombinant IL-2 from such an RP-HPLC pool is to render the mixture aqueous by resuspending (diluting) the pool in an aqueous buffer containing a detergent, such as SDS or sarcosyl, that enhances the solubility of the recombinant IL-2 in water. Following this dilution the organic phase is removed from the recombinant IL-2 containing aqueous phase and the detergent concentration is reduced by diafiltration using an appropriate buffer. When SDS is used, the SDS is reduced to a level of about 100 to 250, preferably approximately 200,  $\mu\text{g}/\text{mg}$  IL-2. Following diafiltration, the IL-2 concentration is readjusted to a concentration in the range of about 0.01 to 2 mg/ml and the water soluble carrier is added to the desired level. The carrier will typically be added such that it is present in the solution of about 1 to 10% by weight, preferably about 5% by weight. The exact amount of carrier added is not critical. Conventional solid bulking agents that are used in pharmaceutical tablet formulation may be used as the carrier. These materials are water soluble, do not



react with the IL-2, and are themselves stable. They are also preferably non-sensitive (i.e., nonhygroscopic) to water. Examples of carriers that may be added are lactose, mannitol, and other reduced sugars such as sorbitol, starches and starch hydrolysates derived from wheat, corn, rice, and potato, microcrystalline celluloses, and albumin such as human serum albumin. Mannitol is preferred.

The carrier adds bulk to the formulation such that when unit dosage amounts of the solution are lyophilized in containers, such as sterile vials, the freeze-dried residue will be clearly discernible to the naked eye. In this regard the preferred carrier, mannitol, yields an aesthetically acceptable (white, crystalline) residue that is not sensitive to water. The nonsensitivity of mannitol to water may enhance the stability of the formulation.

After adding the carrier the unit dosage amounts (i.e., volumes that will provide 0.01 to 2 mg, preferably 0.2 to 0.3 mg, IL-2 per dose) of the solution are dispensed into containers, the containers are capped with a slotted stopper, and the contents are lyophilized using conventional freeze-drying conditions and apparatus.

The lyophilized, sterile product consists of a mixture of (1) recombinant IL-2, (2) carrier (mannitol), (3) detergent (SDS), and (4) a small amount of buffer that will provide a physiological pH when the mixture is reconstituted. The recombinant IL-2 will typically constitute about 0.015% to 3.85% by weight of the mixture, more preferably about 0.4% to 0.6% of the mixture. Storage tests of this product indicate that the IL-2 is stable in this form for more than three months at 2.degree. C. to 8.degree. C.

The lyophilized mixture may be reconstituted by injecting a conventional parenteral aqueous injection such as water for injection, Ringer's injection, dextrose injection, dextrose and salt injection, or the like, into the vial. The injection should be added against the side of the vial to avoid excess foaming. The amount of injection added to the vial will typically be in the range of 1 to 5 ml, preferably 1 to 2 ml.

The reconstituted formulation is suitable for parenteral administration to humans or other mammals to provide IL-2 therapy thereto. Such therapy is appropriate for a variety of immunomodulatory indications such as T cell mutagenesis, induction of cytotoxic T cells, augmentation of natural killer cell activity, induction of IFN-gamma, restoration or enhancement of cellular immunity (e.g., treatment of immune deficient conditions), and augmentation of cell mediated anti-tumor activity.

The following example further illustrates the invention. This example is not intended to limit the invention in any manner.

#### EXAMPLE

The recombinant IL-2 used in this example is des-ala IL-2.sub.ser125. The amino acid sequence of this IL-2 differs from the amino acid sequence of native human IL-2 in that it lacks the initial alanine of the native molecule and the cysteine at position 125 has been changed to serine. Samples of E. coli that produce this IL-2 have been deposited by Cetus Corporation in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA, on Sept. 26, 1983 under accession No. 39452 and on Mar. 6, 1984 under accession number 39626 under the provisions of the Budapest Treaty.

329 mg of an RP-HPLC purified cysteine oxidized IL-2 product (protein concentration 0.94 mg/ml) in 60% 2-propanol, 6% acetic acid was diluted ten-fold into 50 mM sodium acetate, 1 mM ethylene diamine tetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS) at pH 5.5.

The recombinant IL-2 solution was then concentrated using a 10 sq. ft. hollow fiber cartridge (nominal molecular weight cut-off 10,000 daltons) to a volume of 600 ml and then diafiltered for 3 volumes

against 50 mM sodium acetate, 1 mM EDTA, 0.1% SDS at 5.5. The material was then further diafiltered against 10 mM sodium phosphate containing 5 .mu.g SDS/ml until the residual SDS reached a value of 131 .mu.g SDS/mg protein. Approximately 255 mg IL-2 at a concentration of 0.6 mg/ml were recovered (425 ml).

Only 222 mg were used for the formulation which was carried out as follows: 370 ml of the IL-2 solution (222 mg, 0.6 mg/ml) was diluted with 10 mM sodium phosphate, pH 7.5 and 20% mannitol such that the final composition was:

\_\_\_\_\_ 0.25 mg/ml IL-2 in 10 mM sodium phosphate, pH 7.5  
5% mannitol \_\_\_\_\_

The solution was then sterile filtered through a 0.2 micron filter, filled into sterile vials (1.2 ml fill volume) and lyophilized. The product was sealed under vacuum.

The thus produced formulation has been used clinically in humans and has been well tolerated at dosages up to 2 million units/m.sup.2 when administered as a continuous intravenous infusion or up to 1 million units/m.sup.2 when administered as an intravenous or intramuscular bolus. Suitable indications for use of the recombinant IL-2 include:

- (1) treatment of immunodeficiency states, acquired, inborn, or induced by chemotherapy, immunotherapy, or irradiation;
- (2) enhancement of cell-mediated immune responses in the therapy of viral, parasitic, bacterial, malignant, fungal, protozoal, or mycobacterial or other infectious diseases;
- (3) induction of enhanced immunologic response of cells ex vivo in the treatment of infectious, malignant, rheumatic, or autoimmune diseases;
- (4) treatment of rheumatoid or other inflammatory arthritides;
- (5) treatment of diseases of abnormal immune response such as multiple sclerosis, systemic lupus erythematosus, glomerulonephritis, or hepatitis;
- (6) regulation of hematopoietic tumors or pre-malignant or aplastic abnormalities of hematopoietic tissue;
- (7) use as an adjuvant in induction of cell-mediated or humoral response to naturally occurring, administered natural, chemically synthesized or modified, or recombinantly engineered vaccines or other antigens administered for therapeutic purposes;
- (8) use as a mediator of neurotransmission or as a psychoactive therapeutic, as an enkephalin for therapeutic purpose, or as a modifier of central nervous system function;
- (9) in a topical application for the treatment of above-mentioned disease states;
- (10) in combination with cytotoxic chemotherapy or irradiation or surgery in the treatment of malignant or pre-malignant diseases in a direct therapeutic or adjuvant setting;
- (11) in combination with agents with direct anti-viral, anti-fungal, anti-bacterial, or anti-protozoal activity or in combination with drug therapy for typical and atypical m. tuberculosis;

(12) in combination with other immune-modulating drugs, lymphokines, (e.g., IL-1, IL-3, CSF-1, alpha-interferons, and gamma-interferons) naturally occurring or inducible anti-cellular toxins or molecules which mediate lysis or stasis of malignant cells in the treatment of malignant, infectious, autoimmune, or rheumatic diseases; and

(13) for prophylaxis against infectious diseases.

In a similar manner, recombinant IL-2 proteins of the wild-type as disclosed in European patent publication No. 91,539 or the oxidation resistant muteins wherein methionine(s) has been replaced by another amino acid disclosed in U.S. Ser. No. 692,596, filed Jan. 18, 1985, the disclosure of which is incorporated herein by reference, may be formulated in accordance with the present invention. All forms of IL-2, whether of the wild-type or native form or muteins thereof, are contemplated to be within the scope of the present invention.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in the field of pharmaceutical formulation or related fields are intended to be within the scope of the following claims.

\* \* \* \* \*

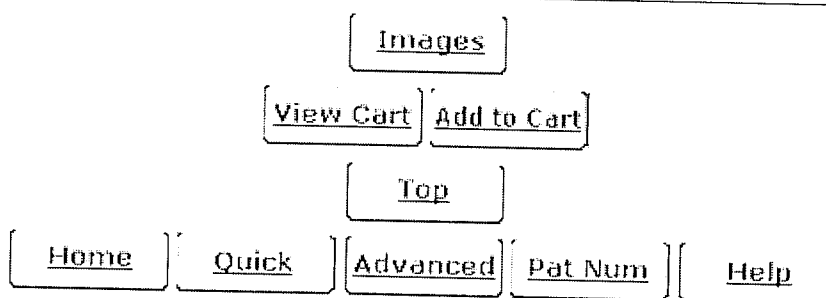


EXHIBIT N  
PATENT 4,748,234



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( 1 of 1 )

**United States Patent****4,748,234****Dorin , et al.****May 31, 1988**

Process for recovering refractile bodies containing heterologous proteins from microbial hosts

**Abstract**

A refractile material containing a heterologous protein is recovered from a host microorganism cell culture transformed to produce the protein. One recovery process involves disrupting the cell wall and membrane of the host cell, removing greater than 99% by weight of the salts from the disruptate, redispersing the desalted disruptate, adding a material to the disruptate to create a density or viscosity gradient in the liquid within the disruptate, and separating the refractile material from the cellular debris by high-speed centrifugation. Another version of such a recovery process comprises the further steps of solubilizing the refractile material under reducing conditions, organically extracting the solubilized refractile material, and isolating said refractile material from the extractant. Preferably the protein is recombinant IL-2 or IFN-.beta. and the salt removal step is carried out by diafiltration.

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Appl. No.: **06/843,997**

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**Related U.S. Patent Documents**

<u>Application Number</u>	<u>Filing Date</u>	<u>Patent Number</u>	<u>Issue Date</u>
749951	Jun., 1985		

**Current U.S. Class:**

**530/412** ; 435/69.51; 435/69.52; 530/351; 530/414; 530/416;  
530/417; 530/422; 530/424; 530/825

**Current International Class:**

C07K 1/00 (20060101); C07K 1/36 (20060101); C07K  
1/113 (20060101); C07K 14/435 (20060101); C07K

14/565 (20060101); C07K 14/55 (20060101); C07K  
003/12 (); C07K 003/28 (); A61K 045/02 ()

**Field of Search:** 530/351,412,414,416,417,422,424,825 435/68,70 424/85,811

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#### Parent Case Text

This application is a continuation-in-part application of U.S. patent application Ser. No. 749,951, filed June 26, 1985 now abandoned.

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*Claims*

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What is claimed is:

1. A process for recovering a refractile material containing a heterologous protein from a host microorganism cell culture transformed to produce said protein, said process comprising:
  - (a) disrupting the cell wall and cell membrane of the microorganism;
  - (b) removing greater than 99% by weight of the salts from said disruptate;
  - (c) redispersing the desalted disruptate;
  - (d) adding a material to the disruptate to increase the density or viscosity of, or to create a density or viscosity gradient in, the liquid within the disruptate; and
  - (e) separating the refractile material from the cellular debris by high-speed centrifugation.
2. The process of claim 1 wherein step (a) is accomplished in the presence of 1-octanol.
3. The process of claim 2 further comprising the step of adding distilled water to the disruptate after step (a) and during step (b).
4. The process of claim 3 wherein step (b) is accomplished by diafiltration.
5. The process of claim 1 wherein step (b) is accomplished by centrifuging the cell membrane and resuspending in deionized water.
6. The process of claim 1 wherein step (d) is accomplished by adding a material to increase the density of the liquid to a  $\rho$  of from about 1.1 to 1.3 g/cm<sup>3</sup>.
7. The process of claim 6 wherein the density of the liquid is increased to a  $\rho$  of from 1.13 to 1.17 g/cm<sup>3</sup>.
8. The process of claim 7 wherein the material is one or more sugars.
9. The process of claim 8 wherein the sugar is sucrose.
10. The process of claim 9 wherein a mixture of sucrose and glycerol is added to form a two-phase system.
11. The process of claim 1 wherein step (d) is accomplished by adding a material to increase the viscosity of the liquid to between 5 and 10 cps.
12. The process of claim 1 wherein the protein is interferon- $\beta$  or interleukin-2.

13. The process of claim 1 wherein the protein is interleukin-2.
14. The process of claim 13 wherein the interleukin-2 is des-ala-ser.sub.125 IL-2.
15. The process according to claim 1 further comprising the following steps:
  - (f) solubilizing the refractile material under reducing conditions;
  - (g) organically extracting the solubilized refractile material; and
  - (h) isolating said refractile material from the extractant.
16. The process according to claim 15 wherein step (f) is accomplished with a solubilizing agent in an aqueous buffer in the presence of a reducing agent.
17. The process of claim 16 wherein the solubilizing agent is SDS and the aqueous buffer is phosphate buffered saline.
18. The process according to claim 16 wherein the percentage range of the solubilizing agent is 1 to 5%.
19. The process according to claim 17 wherein SDS is at a concentration of approximately 2%.
20. The process according to claim 15 wherein step (f) is carried out with DTT as the reducing agent in a concentration of from 5 to 20 mM.
21. The process according to claim 20 wherein DTT is at a concentration of 10 mM.
22. The process according to claim 15 wherein step (f) is carried out in the presence of a chelating agent in a concentration range of between 1 and 5 mM.
23. The process according to claim 22 wherein the chelating agent is EDTA in a concentration of approximately 2 mM.
24. The process according to claim 15 wherein step (f) is carried out at an alkaline pH ranging from about 8.5 to about 9.5.
25. The process according to claim 24 wherein the pH is approximately 9.
26. The process according to claim 15 wherein the reducing conditions of step (f) include an elevated temperature of between 45.degree.-55.degree. C.
27. The process according to claim 15 comprising the additional step of adjusting the pH to between 7 and 7.8 before beginning the organic extraction of step (g).
28. The process of claim 15 wherein step (g) is accomplished by using 2-butanol as the organic extractant.
29. The process of claim 28 wherein the volume ratios of the extractant to the suspension are in a range of about 0.8:1 to about 3:1.

30. The process of claim 28 wherein said volume ratios are equimolar.
31. The process of claim 15 wherein step (g) is accomplished by employing an acid precipitation step followed by centrifugation.
32. The process of claim 31 wherein the acid precipitation step is accomplished by adjusting the pH to the range of from 5 to 6.5.
33. The process of claim 32 wherein the pH is adjusted to 6.2+-.0.1 with glacial acetic acid.
34. The process of claim 31 wherein the centrifugation is carried out at between 10,000 and 15,000 xg.
35. The process of claim 34 wherein the centrifugation is carried out for from 2 to 6 hours.
36. The process of claim 31 wherein the acid precipitation is accomplished in the presence of a solubilizing agent in an aqueous buffer and in the presence of a reducing agent.
37. The process of claim 36 wherein the solubilizing agent is SDS, the aqueous buffer is phosphate buffered saline and the reducing agent is DTT.
38. The process according to claim 15 wherein the protein is interferon-.beta. or interleukin-2.
39. A process for recovering a refractile material containing recombinant IL-2 or recombinant IFN-.beta. from E. coli transformed to produce it, said process comprising:
- (a) concentrating the host E. coli cells by cross-flow filtration;
  - (b) disrupting the cell wall and cell membrane of the E. coli by a disrupting means in the presence of 1% 1-octanol;
  - (c) adding distilled water to the disruptate;
  - (d) diafiltering the disruptate until greater than 99% of the salts are removed using continuous addition of distilled water such that the rate of addition of water equals the diafiltration rate;
  - (e) redispersing the desalted disruptate;
  - (f) adding sucrose to increase the density of the liquid to a .rho. of between 1.13 and 1.17 g/cm.sup.3 ; and
  - (g) separating the refractile material from the cellular debris by high speed centrifugation.
40. The process of claim 39 further comprising the steps of (h) solubilizing the IL-2 or IFN-.beta. in the refractile material with an aqueous solution of a solubilizing agent which forms a water-soluble complex with the IL-2 or IFN-.beta., said solution containing a reducing agent; (i) separating the IL-2 or IFN-.beta. from the resulting solution in the presence of the reducing agent; (j) oxidizing the product of step (i); and (k) purifying the oxidized product by reverse-phase high performance liquid chromatography.
41. The process of claim 40 wherein said solubilizing agent is sodium dodecyl sulfate or sodium laurate sarcosine, the reducing agent is dithiothreitol, step (i) is carried out by gel filtration or reverse-phase high performance liquid chromatography, and step (j) is carried out using iodosobenzoic acid.

42. The process of claim 41 wherein step (i) is carried out by isolating an IL-2 or IFN-.beta. containing fraction from the solution by gel filtration and purifying the resulting IL-2 or IFN-.beta. from the fraction by reverse-phase high performance liquid chromatography, and after step (k) the purified product is formulated and lyophilized.

43. The process of claim 39 further comprising the following steps:

(h) solubilizing the refractile material from step (g) under reducing conditions;

(i) extracting the solubilized refractile material with 2-butanol; and

(j) isolating the extracted refractile material by acid precipitation followed by centrifugation.

44. The process of claim 43 further comprising the steps of (k) solubilizing the extracted refractile material from step (j) under reducing conditions and at an alkaline pH; (l) adjusting the pH to approximately 5.5; (m) separating the IL-2 or IFN-.beta. from the solution in the presence of a reducing agent; (n) oxidizing the product of step (m); (o) purifying the oxidized product by protein purification procedures.

45. A process according to claim 44 wherein step (n) is carried out using iodosobenzoic acid and step (o) is carried out by gel filtration, reverse-phase high performance liquid chromatography (RP-HPLC) or by a combination of gel filtration and RP-HPLC.

46. The process of claim 45 wherein the purified product from step (o) is formulated and lyophilized.

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*Description*

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## BACKGROUND OF THE INVENTION

This invention relates to a biochemical separation or recovery process in which refractile bodies containing microbially produced proteins are separated or recovered from the microorganism hosts which produce them.

Many types of proteins, particularly proteins of potential therapeutic use such as interferon (IFN), interleukin-2 (IL-2), feline leukemia virus antigen (FeLV), etc. have been produced from transformed host cells containing recombinant DNA. The host cells are transformed with expression vectors containing genes encoding the desired heterologous protein and are then cultured under conditions favoring production of the desired protein.

Often the heterologous protein produced by the host cell precipitates inside the cell as opposed to being soluble in the cell. The intracellularly produced protein must be separated from the cellular debris and recovered from the cell before it can be formulated into a purified biologically active material.

Procedures for purifying native IL-2 from T cells are described by Watson, J. et al., J. Exp. Med. (1979) 150: 849-861; Gillis, S. et al., J. Immunology (1980) 124: 1954-1962; Mochizuki, D. Y., et al., J. Immunol. Meth. (1980) 39: 185-201; Welte, K. et al., J. Exp. Med. (1982) 156: 454-464; EP No. 92,163 and EP No. 94,317. In general, these procedures involve precipitating proteins from culture supernatants with ammonium sulfate followed by a chromatographic fractionation.



U.S. Pat. Nos. 4,450,103 and 4,462,940 and Derynck, R., et al., Nature (1980) 287: 193-197 describe procedures for recovering IFN-.beta. from IFN-.beta.-producing E. coli. The patents describe procedures in which IFN-.beta. is extracted from cellular material with 2-butanol or 2-methyl-2-butanol.

Commonly owned U.S. Pat. No. 4,569,790, filed Mar. 28, 1984 and issued Feb. 11, 1986 to K. Koths et al., describes a process for recovering IL-2 from an IL-2-producing microorganism whereby the microorganism cell membrane is disrupted, the disruptate is extracted with an aqueous solution of a chaotropic agent such as urea, the IL-2 is solubilized with, e.g., sodium dodecyl sulfate, and the IL-2 is separated in the presence of a reducing agent.

Commonly owned U.S. Pat. No. 4,530,787 filed Oct. 17, 1984 and issued July 23, 1985 to Z. Shaked et al., describes a process for oxidizing recombinant proteins such as IL-2 selectively and stoichiometrically using o-iodosobenzoic acid to ensure that the protein will be functionally equivalent to its native counterpart.

The above techniques for recovering the protein generally require use of costly reagents which must be removed from the protein prior to formulation thereof. Furthermore, many heterologous proteins are precipitated intracellularly in the form of refractile or inclusion bodies which appear as bright spots visible within the enclosure of the cell under a phase contrast microscope at magnifications down to 1000 fold. See, e.g., Miller et al., Science (1982) 215: 687-690 Cheng, Biochem. Biophys. Res. Comm., (1983) 111: 104-111. Using the techniques described above, such proteins may not be sufficiently isolated from protein contaminants or from forms of the protein which are biologically inactive when produced intracellularly.

Becker et al., Biotech. Adv. (1983) 1: 247-261 disclose separation of these bodies from most of the cell debris and soluble impurities by a low-speed centrifugation. In addition, Kleid et al., ch. 25 in Developments in Industrial Microbiology, Vol. 25, p. 317-325 (Society for Industrial Microbiology, Arlington, VA, 1984) disclose purification of refractile bodies by homogenization followed by centrifugation. Also, Marston et al., Bio/Technology (September, 1984), pp. 800-804 describe release of inclusion bodies by enzymatic and mechanical disruption procedures as well as sonication. Centrifugation at 12,000 xg for five minutes at 4.degree. C. of the cell lysates removed all the inclusion bodies from the supernatant. The resulting pellets are suspended in Triton X100 and EDTA and centrifuged before denaturation.

Purification and activity assurance of precipitated heterologous proteins is also described by U.S. Pat. Nos. 4,511,502; 4,511,503; 4,512,922; and 4,518,526; and EP No. 114,506. U.S. Pat. No. 4,511,502 describes purifying the refractile protein by isolation thereof from a host cell, dissolving the protein in a strong denaturing solution and removing impurities by high speed centrifugation. U.S. Pat. No. 4,511,503 describes and claims isolating the refractile protein from a host cell and treating the protein with a strongly denaturing solution. U.S. Pat. No. 4,512,922 describes and claims solubilizing the refractile bodies in a strongly denaturing solution and replacing with a weakly denaturing medium. U.S. Pat. No. 4,518,526 describes treating the host cell culture with a buffer of sufficient ionic strength to solubilize the host protein but not the refractile protein, disrupting the cells, and treating the insoluble fraction so as to obtain the refractile protein. EP No. 114,506 discloses a method for treating refractile material containing a heterologous protein so as to recover the protein from its host cell by contacting the refractile material with a denaturing solution which may optionally be contacted with a size-discriminating molecular sieve or subjected to high speed centrifugation to remove high molecular weight components from the solution. The examples of EP No. 114,506 indicate that the process requires repeated runs for successful recovery of product.



There remains a need in the art for a method of recovering refractile material containing heterologous expression products from the host cells which method is less costly, is easier to handle, and results in maximum recovery of pure protein in a biologically active form without use of chemical agents.

## SUMMARY OF THE INVENTION

The present invention relates to a recombinant protein recovery process which does not employ costly reagents with their attendant disposal problems, does not require repeated runs, and results in a pure, biologically active protein product.

More specifically, the present invention relates to a process for recovering a refractile material containing a heterologous protein from a host microorganism cell culture transformed to produce said protein, said process comprising:

- (a) disrupting the cell wall and cell membrane of the microorganism;
- (b) removing greater than 99% by weight of the salts from said disruptate;
- (c) redispersing the desalted disruptate;
- (d) adding a material to the disruptate to increase the density or viscosity of, or to create a density or viscosity gradient in, the liquid within the disruptate; and
- (e) separating the refractile material from the cellular debris by high-speed centrifugation.

In preferred embodiments step (b) is accomplished by diafiltration or centrifugation and step (d) is accomplished by increasing the density or viscosity of the liquid to within certain specified ranges.

The present invention also relates to a process for recovering a refractile material containing a heterologous hydrophobic protein from a host microorganism cell culture transformed to said protein, that encompasses not only steps (a) through (e) as outlined immediately above but also comprises the following steps:

- (f) solubilizing the refractile material under reducing conditions;
- (g) organically extracting the solubilized refractile material; and
- (h) isolating said refractile material from the extractant.

In preferred embodiments, step (f) is accomplished with a solubilizing agent in an aqueous buffer in the presence of a reducing agent; step (g) is accomplished by using 2-butanol as the organic extractant; and step (h) is accomplished by employing an acid precipitation step followed by centrifugation.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the general scheme used in Examples I-IV to isolate the heterologous protein.

FIG. 2 depicts an alternative general scheme, as used in Example V, to isolate a heterologous protein.

FIG. 3 is an SDS-PAGE reducing gel showing the amount of interleukin-2 obtained at various stages of the process of this invention.

FIG. 4 is a flow diagram illustrating the details of each step of one of the preferred entire processes from fermentation of the host culture to lyophilization of the formulated purified protein product.

FIGS. 5A and 5B are flow diagrams illustrating the details of each step of another preferred entire process from fermentation of the host culture to lyophilization of the formulated purified protein product. These figures correspond to the procedure followed in Example V.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, the term "heterologous" proteins refers to proteins which are foreign to the host cell transformed to produce them. Thus, the host cell does not generally produce such proteins on its own. Such proteins are produced by recombinant DNA technology using techniques well known in the art. The proteins herein are often also hydrophobic, i.e., they are not soluble or not readily soluble in aqueous medium under ambient conditions of room temperature and atmospheric pressure at a pH of between about 6.5 and 7.8, i.e., at about neutral or physiological pH.

The heterologous proteins are recovered from refractile materials by the present process. The term "refractile" material designates material or bodies which refract light and appear as bright spots when viewed through a phase contrast microscope. Refractile material is also known as "inclusion" bodies. Examples of heterologous proteins which form refractile bodies in commonly found culture conditions include interleukin-2 (IL-2), interferon-.beta. (IFN-.beta.), envelope protein from feline leukemia virus antigen (FeLV), human growth hormone (hGH), bovine growth hormone (bGH), porcine growth hormone (pGH), and certain proteins coated or fused with a virus such as FMD virus. Certain proteins, such as interferon-.alpha. (IFN-.alpha.), interferon-.gamma. (IFN-.gamma.), and tumor necrosis factor (TNF), are more soluble in the cytoplasm.

The precise chemical structure of the protein will depend on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular protein may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their activity when placed in suitable environmental conditions are included in the definition of proteins herein. Further, the primary amino acid sequence of the protein may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like, more commonly by conjugation with saccharides. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced in vitro. In any event, such modifications are included in the definition of protein herein so long as the activity of the protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in the various assays.

Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the protein may be cleaved to obtain fragments which retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the definition.

Finally, modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the activity of the protein. For example, at least one cysteine residue which is not essential to biological activity, is present in the biologically active protein, and is free to form a disulfide link may be deleted or replaced with another amino acid to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation. Such modified proteins, known as "muteins," are described in U.S. Pat. No. 4,518,584 issued May 21, 1985. In another example, a conservative amino acid of a biologically active

protein such as IL-2 or IFN-.beta. is substituted for each methionine residue susceptible to chloramine T or peroxide oxidation, wherein additional, non-susceptible methionine residues are not so substituted. A conservative amino acid alteration in this context is defined as one which does not adversely affect biological activity and involves neutral or non-polar amino acid substitutions or deletion of the methionine. In a preferred example of this embodiment the methionine at amino acid position 104 of IL-2 is replaced by an alanine residue.

Preferably the protein herein is IL-2 or IFN-.beta.. Most preferably the protein is unglycosylated IL-2 which is produced by a microorganism that has been transformed with a human IL-2 gene or a modification of the human IL-2 gene that encodes a protein having: (a) an amino acid sequence that is at least substantially identical to the amino acid sequence of native human IL-2 and (b) biological activity that is common to native human IL-2. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not cause an adverse functional dissimilarity between the synthetic protein and the native human IL-2. Examples of such proteins are the IL-2s described in European patent application No. 83101035.0 filed Feb. 3, 1983 (published Oct. 19, 1983 under publication no. 91539) and European patent application No. 82307036.2 filed Dec. 22, 1982 (published Sept. 14, 1983 under no. 88195), the mutein IL-2s described above, and the IL-2s described in the examples of this application.

As used herein the term "transformed" in describing host microorganism cell cultures denotes a microorganism that has been genetically engineered to produce a heterologous protein that possesses the activity of the native protein. Examples of transformed microorganisms are described in the examples of this application. Bacteria are preferred microorganisms for producing the protein. Synthetic protein may also be made by suitably transformed yeast and mammalian cells. E. coli is particularly preferred.

## FRONT-END PROCESSES

The transformed microorganisms are grown in a suitable growth medium, typically to an optical density (OD) of at least about 30 at 680 nm, and preferably between about 20 and 40 at 680 nm. The composition of the growth medium will depend upon the particular microorganism involved. The medium is an aqueous medium containing compounds that fulfill the nutritional requirements of the microorganism. Growth media will typically contain assimilable sources of carbon and nitrogen, energy sources, magnesium, potassium and sodium ions, and optionally amino acids and purine and pyrimidine bases. (See Review of Medical Biology, Lange Medical Publications, 14th Ed pp. 80-85 (1980).) In expression vectors involving the trp promoter, the tryptophan concentration in the medium is carefully controlled to become limiting at the time protein expression is desired. Growth media for E. coli are well known in the art.

After the cells are harvested from the culture, they may be concentrated, if necessary, to about 20 to 150 mg/ml, preferably 80 to 100 mg/ml (OD 40 to 300, preferably 160 to 200 at 680 nm) by cross-flow filtration, centrifugation, or other conventional methods. Preferably a compound which is non-toxic to humans, such as 1-octanol, in an amount of about 1% by weight of total components, is added to the fermenter before or during cell concentration to ensure that no viable recombinant organisms remain before cell membrane containment is broken.

Following concentration of the harvested culture, the cell membranes of the microorganisms are disrupted. Conventional cell disruption techniques such as homogenization, sonication, or pressure cycling may be used in this step of the process. Preferred methods are sonication or homogenization with a homogenizer. The end point of the disruption step can be determined by monitoring the optical density with the absorbance at 260 nM of the suspension typically increasing with cell lysis. In any event, the disruption should break substantially all of the cells so that substantially no intact cells are

carried through to the solubilization step. Before the disruption, the pH of the liquid phase of the concentrate is adjusted, if necessary, to a level that facilitates removal of E. coli proteins in subsequent steps, while retaining the heterologous protein as an insoluble complex in the cellular debris.

The steps in the recovery process subsequent to the disruption step are primarily designed to separate the refractile material from the other contaminating proteins and other cellular debris. Using the process herein the refractile bodies can be isolated from the cellular debris to obtain a protein purity of about 50% by weight. Subsequent isolation and purification of the protein using the preferred techniques herein will yield a product of at least 95% purity, preferably at least 98% purity, in good yields. Simultaneously, this purification process also reduces pyrogenic substances in the final product to a level believed to be acceptable for parenteral administration to patients.

After the cells have been disrupted, deionized water is preferably added to the disruptate and greater than 99% by weight of the salts are removed therefrom. The salts are water-soluble materials composed of oppositely charged small molecular weight ions. The removal of these salts to reduce the ionic strength of the disruptate may be accomplished by diafiltration using deionized water to flush out the ions or by centrifuging to pellet the cellular debris and refractile bodies followed by resuspension in deionized water. If diafiltration is employed, preferably deionized water is continuously added such that the rate of addition of water equals the filtration rate.

After the salts are essentially removed, optionally a compound such as 1-octanol may be added to the desalted disruptate, if not added earlier, to ensure that no viable recombinant organisms remain before containment is broken. The desalted disruptate is again disrupted as described above for the initial disruption.

After redispersion, density or viscosity is increased and/or a gradient is created during centrifugation in the liquid within the disruptate by adding a material to the disruptate. There are several means to accomplish this purpose, all relying on the sedimentation characteristics of the particles by varying the density and/or viscosity of the liquid phase. One means to accomplish this goal is to add a material which increases the density of the liquid to a  $\rho$  of about 1.1 to 1.3 g/ml, preferably 1.13 to 1.17 g/ml.

Materials which may be used to accomplish this density increase include a sugar or mixture of sugars, such as, e.g., sucrose, dextrose, fructose, maltose, maltotriose, and other mono-, di- or polysaccharides. Most preferably the sugar is sucrose. Alternatively, a two-phase system of materials such as, e.g., a glycerol/sucrose mixture may be used wherein the disrupted particles partition to the interface between the heavy and light phases and can be eluted by a liquid/liquid separation.

In addition, the viscosity of the liquid phase may be increased to from 5 to 10 cps by any suitable means such as by adding a viscous compound such as, e.g., sucrose or glycerol thereto. Also, a gradient is created if, e.g., the particles are in a 60% aqueous glycerol suspension while the centrifuge bowl contains 80% aqueous glycerol.

In the final step of the abbreviated "front-end" process to recover the refractile bodies, the refractile bodies containing the desired protein are separated from the cellular debris by high-speed centrifugation. By "high-speed centrifugation" is meant spinning the suspension in a centrifuge at about 10,000 to 40,000 times gravity, preferably about 10,000-20,000.times.g, for a suitable time period depending on the volume, generally about 10 minutes to seventy-two hours. The density of the medium will generally be too high to separate the particles by low-speed centrifugation. Therefore, if the centrifugation is carried out at low speeds (e.g., at 500 to 5,000.times.g), satisfactory results are not obtained. The exact centrifuge speed will depend on the protein and the final concentration of material added to create the gradient (e.g., sucrose). For example, interferon may require lower sucrose concentrations to obtain



maximal recovery, and thus the centrifugation speed may be lowered, or the centrifuge retention time decreased.

FIG. 1 illustrates one preferred scheme for obtaining the desired protein contained within the refractile bodies. In this scheme the cells containing refractile bodies are concentrated by cross-flow filtration and disrupted. Then the disruptate is diafiltered against deionized water to reduce the ionic strength of the liquid and then redispersed. Sucrose is then added to obtain a final density of the liquid of  $\rho = 1.1$  to 1.3. The mixture is then centrifuged using high-speed centrifugation to obtain a pellet containing the refractile bodies and a supernatant which is discarded. This pellet can be differentiated as the "particle pellet" or "particle paste" from that of the "final" pellet or "final paste" resulting from the alternative, expanded "front-end" process, described below.

An alternative, expanded "front-end" process to recover the refractile bodies is schematically illustrated in FIG. 2. In this scheme, the particle pellet obtained from the centrifugation step (6) in FIG. 1 is solubilized, reduced and then extracted from the aqueous medium with 2-butanol. The extractant phase is then precipitated with an acid and centrifuged to produce a "final pellet" or "final paste" which is then further purified as indicated. Example V exemplifies the expanded front-end process.

The particle pellet resulting from the centrifugation at the end of the abbreviated front-end process contains approximately 15-70% by weight of the desired heterologous protein as determined by Lowry assay [Lowry et al., J. Biol. Chem. (1951) 193: 265-275]. The final pellet from the expanded front-end process contains about 70-85% by weight of the desired protein.

The alternative, expanded front-end process is distinguished from the abbreviated front-end process in that it comprises several additional steps as follows: solubilizing the refractile bodies under reducing conditions; organically extracting the solubilized refractile material; and isolating said refractile material from the extractant. These alternative front-end steps can be done to promote recovery of any of the above-mentioned candidate heterologous proteins that are deposited within the host strain in refractile bodies. Essentially, the enhanced purity of the final pellet as opposed to the particle pellet lessens the purifying burden of downstream processing. There is an interdependence between the choice of the front-end process and later process purification steps to achieve the desired purity level for the final product. Once the choice of the particular front-end recovery of the refractile bodies has been made, one skilled in the art can pick and choose the alternative purifying steps outlined below to achieve the desired purity level of the final product.

The organic extraction of the expanded front-end primarily effects a partition of the hydrophobic proteins from the lipopolysaccharides and nucleic acids of the aqueous and solid phases. Secondly, said extraction also removes some of the host cell's endotoxins and other proteins. The isolation step, preferably by acid precipitation followed by centrifugation, separates the refractile material from the organic extractant and other cellular debris.

For solubilizing the particle pellet of the expanded front-end, the following solubilizing agents can be used: sodium dodecyl sulfate (SDS), sodium laurate, urea, sodium dodecyl sulfonate, sodium decyl sulfate, sodium tetradecyl sulfate, sodium tridecyl sulfonate, sodium myristate, sodium caprate, sodium dodecyl N-sarcosinate, and sodium tetradecyl N-sarcosinate. Preferred solubilizing agents are SDS or sodium laurate. Most preferred is SDS.

The solubilizing agent is in an aqueous buffer, preferably phosphate buffered saline. The preferred percentage of the solubilizing agent is in the range of 1% to 5% (w/v). (Percentages herein reflect weight to volume ratios.) The most preferred solubilizing solution is phosphate buffered saline with 2% SDS.

Reducing agents that can be employed during the solubilization step include: mercaptoethanol, glutathione, cysteine and dithiothreitol (DTT). DTT is the most preferred reducing agent. The concentration of the reducing agent in the medium will usually range between about 5 to 20 mM with approximately 10 mM being the most preferred concentration.

Reduction conditions may also include the addition of a chelating agent such as ethylenediaminetetraacetic acid (EDTA) in concentrations ranging between 1 and 5 mM with approximately 2 mM being the most preferred concentration. It is also preferable to carry out the reduction at an alkaline pH usually ranging between 8.5 and 9.5 with a pH of 9 being especially preferred. The pH adjustment may be accomplished by the addition of a base such as NaOH.

Further, it is preferable to carry out the reduction reaction at an elevated temperature, preferably at 45.degree.-55.degree. C. and most preferably 50.degree. C., under nitrogen to ensure efficient reduction of the material. The reaction will typically be run from between 5 and 15 minutes, most preferably for 10 minutes.

After the reduction is complete, it is usually cooled to approximately 25.degree. C. and the pH adjusted with an acid, preferably glacial acetic acid, to a pH of between 7 and 7.8, most preferably 7.4. Once said solubilization and reduction steps are complete, the organic extraction is begun.

The organic extractant can be 2-butanol, 2-methyl-butanol or mixtures thereof. Most preferably the extractant is 2-butanol. The conditions for extraction would be those that maintain phase separation between the aqueous medium and the extractant.

The extractant will normally be combined with the aqueous solution of the protein in volume ratios in the range of about 0.8:1 to about 3:1, preferably about 1:1 (extractant: volume of the suspension). The extraction may be carried out using conventional batch or continuous liquid-liquid extraction techniques and equipment. The extraction may be carried out at 20.degree. C. to 100.degree. C. and involve contact times in the range of about one minute to one hour.

Upon completion of the extraction, the aqueous phase and extractant phase are separated, and then the desired protein is isolated from the extractant phase. Various isolation techniques such as precipitation, molecular sieve chromatography, affinity chromatography, and electrophoresis can be employed.

A preferred isolation technique is an acid precipitation step followed by centrifugation. The extracted refractile material containing the desired heterologous protein is precipitated from the extractant by mixing the extractant solution with an aqueous buffer containing a solubilizing agent in a phosphate buffered saline under reducing conditions. The preferred solubilizing agent is SDS at a concentration of from about 0.05 to 0.2%, most preferably at 0.1% .

The reducing agent added to the organic extract/buffer solution may be mercaptoethanol, glutathione, cysteine or dithiothreitol (DTT), DTT being the most referred. The final concentration of the reducing agent can be in the range of 1 mM to 5 mM with 2 mM of DTT being the most preferred.

The pH of the organic extract/buffer solutions is then reduced with an acid typically to the range of about 5 to 6.5. This pH adjustment is most preferably carried out with glacial acetic acid to a pH of about 6.2.

The precipitated mixture is then centrifuged at a high speed, preferably at 10,000 to 15,000.times.g, preferably for a time period ranging from 15 minutes to 10 hours, depending upon the size of the fermentation run. For a 1000-liter run, as exemplified in Example V, the preferred centrifuging time



would be 10 hours.

Such centrifuging is the last step of the expanded front-end process resulting in the final pellet or final paste. Whether the abbreviated front-end or expanded front-end are the choice for recovering refractile bodies containing the desired heterologous protein, the next step in purification would be solubilization of the refractile bodies as noted below.

#### DOWNSTREAM PROCESSING

After the last centrifugation step of either the abbreviated front-end process or the expanded front-end process, a pellet, particle or final, is the result containing refractile material at different loads of purity, 15-70% or 70-85% purity, respectively, of the desired heterologous protein. Whether the abbreviated or expanded version of the front-end process, the next step in the purification process is the solubilization with a denaturant of either the particle pellet or the final pellet.

The pellets containing the refractile bodies obtained after centrifugation are preferably solubilized by contact with a neutral aqueous buffer containing not only the protein denaturant (solubilizing agent) but also a reducing agent. Surface active agents (detergents) which have a suitable hydrophobic-hydrophilic balance to solubilize the hydrophobic protein may be used as solubilizing agents. Strong protein denaturants such as alkali metal sulfates containing 10 to 14 carbon atoms and alkali metal alkyl sarcosinates are preferred solubilizing agents, with SDS and sarcosyl being particularly preferred. Optionally, said aqueous buffer can also contain a chelating agent in a concentration of from 3 to 7 mM. Most preferably, said chelating agent would be EDTA at a concentration of 5 mM.

The amount of solubilizing agent used in the solubilization will depend upon the particular agent. When SDS or sarcosyl is used, the preferred concentration (w/v) of SDS/sarcosyl is 1-10% in buffer such as phosphate buffered saline (50 mM sodium phosphate, pH 7, 0.9% sodium chloride). Preferably the range of SDS would be from 2 to 7%, most preferably 5%. The solubilizing medium may also contain a sufficient amount of reducing agent to prevent the solubilized protein from undergoing oxidation to any significant degree. Protein reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol may be used for this purpose. The concentration of reducing agent such as DTT in the medium will usually range between about 5 to 30 mM, most preferably 20 mM. The solubilization will typically be carried out at temperatures in the range of 20.degree. C. to 25.degree. C. with mixing to facilitate contact between the solid phase and the solubilizing medium. Optionally, a reduction step may be carried out at this point. The pH, if necessary, may be adjusted to a range of 8 to 9, most preferably approximately 8.5. The suspension may be heated to 50.+-.5.degree. C. for 5 to 15 minutes under nitrogen. The reaction mixture would then be cooled to approximately 25.degree. C.

The solubilization is considered complete when the sample has sat 15 minutes or the solution turns translucent. Optionally at this point, the insoluble material may be separated by centrifugation or filtration after completing the solubilization.

After the protein is solubilized, the resulting suspension may optionally be centrifuged at 10,000-40,000.times.g, preferably 25,000 to 35,000.times.g, to obtain a pellet containing, inter alia, additional host (e.g., E. coli) proteins, notably including certain contaminants that have molecular weights very close to that of the desired protein. The exact speed of centrifugation is not critical, as most of the insoluble material will come out, even at low speeds. The pellet is discarded and the supernatant containing the desired protein is retained and processed to recover the desired protein. Otherwise, after the solubilization or solubilization/reduction step, the pH of the suspension can be adjusted to a pH of approximately 5 to 6, most preferably 5.5 with glacial acetic acid, and then filtered.

If a reduction step was not carried out during the solubilization, the next step in the process would be a reduction of the solubilized refractile body protein. A preferred reducing agent is dithiothreitol (DTT) which for this purpose may be added to a final concentration ranging from 10 to 100 mM, most preferably from 20 to 50 mM. Reduction conditions may also include the addition of a chelating agent such as ethylenediaminetetraacetic acid (EDTA) in concentrations ranging between 1 and 5 mM. It is also preferable to carry out the reduction at a somewhat alkaline pH usually ranging between 8 and 9.5, with a pH of 8.5+-.0.1 being especially preferred. This pH adjustment may be accomplished by the addition of a base such as NaOH. Furthermore, it is also preferable to carry out the reduction reaction at an elevated temperature under nitrogen to ensure efficient reduction of the material. The reaction will typically be run at 45.degree. to 55.degree. C. for 5 to 30 minutes under nitrogen. Especially preferred is a reaction time of from 10 to 20 minutes. After the reduction is complete it is usually cooled to about 25.degree. C. and the pH is adjusted to a range of 5 to 6 using an acid such as glacial acetic acid. Most preferably the pH would be adjusted to 5.5.

The next step in the process is to separate the protein in the supernatant from any host contaminants remaining after the centrifugation or filtration and optimally from the solubilizing agent. Gel filtration chromatography, reverse-phase high performance liquid chromatography (RP-HPLC), or a combination of gel filtration chromatography and RP-HPLC, can be used. The gel filtration chromatography is preferably carried out in two stages that remove both pyrogenic components and protein contaminants having molecular weights higher or lower than that of the protein. Gels that are capable of fractionating the solution to permit separation of the protein from these contaminants are commercially available. Sephacryl.RTM.S-200 is a preferred gel for removing the higher molecular weight components and Sephadex.RTM.G-25, G-75 or G100 gels are preferred for removing the low molecular weight contaminants. The gel filtrations will typically be run in buffered solutions (pH 5.5 to 7.0) containing about 0.1% to 1.5% solubilizing agent and about 0.5 to 10 mM reducing agent. The column will be sized to permit suitable resolution of the desired components.

RP-HPLC is an alternative to gel filtration. Also, RP-HPLC is capable of removing molecules from the solution that have molecular weights close to the protein and cannot, therefore, be removed completely by gel filtration. In addition, contaminants such as bacterial endotoxin are also removed effectively by RP-HPLC. Therefore, RP-HPLC may also be used as a final purification step after gel filtration. U.S. Pat. No. 4,569,790 to K. Koths et al. discloses the following preferred materials and methods for the purification of IL-2 by RP-HPLC. Supports (stationary phases) that provide good resolution of proteins may be used in the RP-HPLC. C-4, C-8, or C-18 on 300 angstrom pore-size supports are examples of preferred supports. The separation is carried out at a pH of approximately 5.5, in order to keep the protein in solution. In this regard, the pH of the protein solution will preferably be adjusted to this range. The solution is loaded into the RP-HPLC column and is adsorbed onto the stationary phase. A gradient solvent system comprising an organic acid such as acetic acid or trifluoroacetic acid and organic solvent such as propanol or acetonitrile is used to elute the protein from the column. Acetic acid-propanol, trifluoroacetic acid-propanol, and trifluoroacetic acid-acetonitrile are preferred solvent systems. IL-2 elutes in the acetic acid-propanol system at about 40% propanol, in the trifluoroacetic acid-propanol system at about 50% propanol, and in the trifluoroacetic acid-acetonitrile system at about 62% acetonitrile. For convenience, the organic solvent content of the eluant will usually be increased rapidly to a level somewhat below the solvent concentration at which the protein elutes followed by a slow gradient change in the range of about 0.1% to 1.0%/min.

As soon as the protein is recovered from the chromatography step, it is lyophilized and resuspended in a neutral aqueous buffer containing the reducing agent (to keep the protein in a reduced state) and the solubilizing agent (to keep it in solution). The IL-2 is stable in this form and may be stored for further treatment and formulation before being used.

An alternative and preferred procedure is to oxidize selectively, under controlled conditions, the protein after it has been separated by gel filtration, as described in U.S. Pat. No. 4,572,798 filed Dec. 6, 1984 and issued Feb. 25, 1986 to K. Kothe et al. (using an oxidation promoter containing a Cu.sup.+2 cation) and in U.S. Pat. No. 4,530,787 filed Oct. 17, 1984 and issued July 23, 1985 to Z. Shaked et al. (using o-iodosobenzoic acid), the disclosures of which are incorporated herein by reference and described hereinbelow, and purify the oxidized product by RP-HPLC or gel filtration followed by RP-HPLC. Preferred oxidizing agents for this purpose are CuCl.sub.2 and o-iodosobenzoic acid. The Cu.sup.+2 oxidation comprises reacting an aqueous solution containing a solubilized form of the recombinant protein at a pH between about 5.5 and 9 in the presence of air with at least an effective amount of an oxidation promoter containing a Cu.sup.+2 cation. Controlled oxidation causes the formation of disulfide bridging in the recombinant protein which conforms to the bridging in its native counterpart with no or minimal overoxidation and formation of nonconforming bridging or oligomers. Such oxidation enables the production of high yields of the recombinant protein in a configuration that most closely resembles the configuration of its native counterpart, thereby ensuring the likelihood that the recombinant protein will be functionally equivalent to the native protein.

The amount of oxidant or oxidation promoter employed is at least an effective amount for oxidation, i.e., an amount which at minimum will be necessary to conduct the oxidation reaction effectively within a convenient period of time. An effective amount is the amount approximately equivalent to the concentration of free sulfhydryl groups on the protein which are destined to be involved in forming the desired disulfide bonds. Preferably, the amount of CuCl.sub.2 will range from about 1 to 400 micromolar, depending on the protein concentration, more preferably 5 to 50 micromolar if the protein is IL-2. In the case of o-iodosobenzoic acid the mole ratio of oxidant to protein will preferably be in the range of about 0.05:1 to about 5:1, most preferably about 0.8:1 to about 1:2. The concentration of protein in the reaction mixture is kept low, i.e., generally less than about 5 mg/ml, preferably about 0.05 to about 2 mg/ml, and more preferably about 0.1 to about 1 mg/ml, to reduce the likelihood of oligomer formation.

The pH of the reaction medium for Cu.sup.+2 oxidation is generally maintained at a level between about 5.5 and 9, preferably 6 and 8, and more preferably about 7.

The pH of the reaction medium for o-iodosobenzoic acid is maintained at a level at least about one-half pH unit below the pK.sub.a of the cysteine residues being oxidized. When the pK.sub.a's of these residues differ, the pH is preferably maintained at least about one-half pH unit less than the pK.sub.a of the cysteine residue having the lowest pK.sub.a. Control of the pH in this manner for o-iodosobenzoic acid oxidation controls the amount of nonionized thiol, thereby controlling the rate of the reaction and favouring the formation of the desired disulfide bridging. For recombinant IFN-.beta. the pH for o-iodosobenzoic acid oxidation is maintained between 6 and 9, preferably 7.0 and 9.0. For recombinant IL-2, it is maintained between 5.5 and 9, preferably 7.0 and 8.0.

The reduced, cloned protein, which is less soluble than the oxidized form of the protein, generally must remain in solution, i.e., be in solubilized form, for effective oxidation to occur. Therefore, the reaction mixture with Cu.sup.+2 will preferably also contain at least an effective amount of a solubilizing agent to prevent the protein from precipitating out of solution. As used herein, the term "solubilizing agent" refers to an ionic or nonionic protein-solubilizing solute such as, e.g., sodium dodecyl sulfate (SDS) or urea. The amount of solubilizing agent which may be employed for this purpose is generally from about 0.05 to about 1% by weight per volume (for detergents), most preferably 0.1% of about 5-9M (for urea), depending mainly on the protein and types of oxidation promoter used.

The oxidation reaction time will depend, for example, upon the concentration of reagents in the reaction mixture, the reaction temperature, and the types of reagents. The reaction temperature will normally be



between about 20.degree. C. and 40.degree. C., conveniently room temperature, to maintain the solubilizing agent/protein mixture in solution. For Cu.sup.+2 oxidation, increasing the reaction temperature increases the rate of reaction. The oxidation reaction may be effectively terminated by, e.g., lowering the pH to a level at which the reaction ceases, freezing the solution, or adding chelators such as EDTA to the reaction mixture. Following the reaction, residual oxidation promoter and undesired isomers or oligomers may be removed by selective ultrafiltration or chromatographic techniques. If necessary, the oxidized protein may be purified further from side products and any residual reduced protein using protein purification procedures such as reverse phase high performance liquid chromatography (RP-HPLC).

The purity of the protein after the chromatography step(s) is at least about 95% and usually at least about 98%. This highly pure material contains less than about 5 ng endotoxin, usually less than about 0.01 ng endotoxin per 100,000 units protein bioactivity.

The formulation of the protein in accordance with this invention may be carried out as a separate operation using purified, selectively oxidized protein or in an operation that is integrated with the purification of the selectively oxidized protein. In the latter case, the starting material for the formulation is a protein-containing product from a RP-HPLC treatment of the selectively oxidized product. Preferably a product selectively oxidized by the RP-HPLC product (pool) will comprise a solution of the protein in a water-organic solvent mixture. The nature of the organic solvent will depend upon the solvent system used in RP-HPLC. Examples of systems that may be used for the preparation of IL-2 as described in U.S. Pat. No. 4,569,790 to K. Koths et al. are combinations of an organic acid such as acetic acid or trifluoroacetic acid and organic solvent such as propanol or acetonitrile.

Optionally, the first step in one formulation of the protein from such an RP-HPLC pool is to render the mixture aqueous by resuspending (diluting) the pool in an aqueous buffer containing a detergent, such as SDS or sarcosyl, which enhances the solubility of the protein in water. Following this dilution the organic phase is removed from the protein-containing aqueous phase and the detergent concentration is reduced by diafiltration using an appropriate buffer. When SDS is used, the SDS is reduced to a level of about 100 to 250, preferably approximately 200, .mu.g/mg when IL-2 is used as protein. Following diafiltration, the protein concentration is readjusted to a concentration in the range of about 0.01 to 10 mg/ml depending mainly on the protein and its intended use, preferably 0.01 to 2 mg/ml for IL-2, and the water-soluble carrier is added to the desired level. The carrier will typically be added such that it is present in the solution at about 1 to 10% by weight, preferably about 5% by weight. The exact amount of carrier added is not critical. Conventional solid bulking agents that are used in pharmaceutical tablet formulations may be used as the carrier. These materials are water soluble, do not react with the protein, and are themselves stable. They are also preferably non-sensitive to water (i.e., nonhygroscopic). Specific examples of carriers that may be added include dextrose, lactose, mannitol, and other reduced sugars such as sorbitol, starches and starch hydrolysates derived from wheat, corn, rice, and potato, microcrystalline celluloses, and albumin such as human serum albumin. Mannitol and dextrose are preferred.

The carrier adds bulk to the formulation such that when unit dosage amounts of the solution are lyophilized in containers, such as sterile vials, the freeze-dried residue will be clearly discernible to the naked eye. In this regard the preferred carrier, mannitol, yields an aesthetically acceptable (white, crystalline) residue that is not sensitive to water. The nonsensitivity of mannitol to water may enhance the stability of the formulation.

Alternatively, the first step in another preferred formulation of the desired protein is a stabilization step. Alpha-interferons and native beta-interferons are not lipophilic proteins. Therefore, they can be stabilized and solubilized by adding a stabilizer such as human serum albumin at a physiological pH. In

contrast, lipophilic proteins such as recombinant beta-interferon and interleukin-2 are not solubilized by addition of human serum albumin at pH 6.8-7.8.

Copending, commonly owned, U.S. patent application Ser. No. 775,751, filed Sept. 13, 1985, entitled An Improved Formulation for Lipophilic Proteins (Hanisch et al.) outlines an improved process for recovering and purifying lipophilic recombinant proteins such as human .beta.-interferon and interleukin-2 from their hosts to yield a protein preparation which may be formulated into a stable pharmaceutical composition. Such a composition carrying a therapeutically effective amount of the biologically active recombinant lipophilic protein dissolved in a non-toxic, inert, therapeutically compatible aqueous-based carrier medium at a pH of 6.8 to 7.8 also contains a stabilizer for the protein, such as human serum albumin, normal serum albumin and human plasma protein fraction. The formulation aspects of said U.S. Ser. No. 775,751 are herein incorporated by reference as alternative formulation routes for lipophilic proteins which were recovered as refractile bodies by the abbreviated or expanded versions of the front-end processes of the present invention. The 775,751 application outlines a low pH formulation process.

U.S. Pat. No. 4,462,940, filed May 18, 1983, and issued July 31, 1984 to Hanisch et al., outlines a high pH formulation process, and the formulation aspects thereof are herein also incorporated by reference.

After adding the carrier the unit dosage amounts (i.e., for IL-2 volumes that will provide 0.01 to 2 mg, preferably 0.2 to 0.3 mg, IL-2 per dose) of the solution are dispensed into containers, the containers are capped with a slotted stopper, and the contents are lyophilized using conventional freeze-drying conditions and apparatus.

The lyophilized, sterile product consists of a mixture of (1) protein, (2) carrier (dextrose or mannitol), (3) detergent (SDS), and (4) a small amount of buffer that will provide a physiological pH when the mixture is reconstituted. The product may also contain a minor amount of a preservative to enhance chemical stability. If the protein is IL-2, the recombinant IL-2 will typically constitute about 0.015% to 3.85% by weight of the mixture, more preferably about 0.4% to 0.6% of the mixture. Storage tests of this product indicate that the IL-2 is stable in this form for more than three months at 2.degree. C. to 8.degree. C.

The lyophilized mixture may be reconstituted by injecting a conventional parenteral aqueous injection such as distilled water for injection, Ringer's solution injection, Hank's solution injection, dextrose injection, dextrose and salt injection, physiological saline injection, or the like, into the vial. The injection should be added against the side of the vial to avoid excess foaming. The amount of injection added to the vial will typically be in the range of 1 to 5 ml, preferably 1 to 2 ml.

In an alternative formulation, described in copending U.S. Application Ser. No. 749,955, filed June 26, 1985, that is, filed concurrently with the parent application of the instant application (U.S. Ser. No. 749,951), now abandoned in favor of U.S. Ser. No. 866,459, filed May 21, 1986, and entitled "Solubilization of Proteins For Pharmaceutical Compositions Using Homopolymer Conjugation" to M. Knauf et al., the common disclosure of which is incorporated herein by reference, the hydrophobic protein may be solubilized, not by a detergent, but by reacting the protein with an activated homopolymer selected from polyethylene glycol, polypropylene glycol or polybutylene glycol, said homopolymer having a molecular weight of from 500 to 20,000 daltons, preferably 2000 to 10,000 daltons. The homopolymer is activated by conjugation with a coupling agent having terminal groups reactive with both the free amine or thiol groups of the protein and the hydroxyl group of the homopolymer. Examples of such coupling agents include hydroxynitrobenzene sulfonic ester, cyanuric acid chloride, and N-hydroxysuccinimide. This modification eliminates the necessity for adding detergents to solubilize the protein at physiological pH. The protein is then formulated directly with the

water-soluble carrier and buffer as described above, the formulation is lyophilized, and the lyophilized mixture may be reconstituted as described above.

FIG. 4 illustrates a flow diagram of one preferred scheme for obtaining the desired protein, from fermentation to lyophilization. FIGS. 5A and 5B illustrate another preferred scheme.

The reconstituted formulation prepared as described above is suitable for parenteral administration to humans or other mammals in therapeutically effective amounts (i.e., amounts which eliminate or reduce the patient's pathological condition) to provide therapy thereto, the type of therapy being dependent on the type of protein. For example, IL-2 therapy is appropriate for a variety of immunomodulatory indications such as T cell mutagenesis, induction of cytotoxic T cells, augmentation of natural killer cell activity, induction of IFN-gamma, restoration or enhancement of cellular immunity (e.g., treatment of immune deficient conditions), and augmentation of cell-mediated anti-tumor activity. IFN-.beta. therapy is appropriate for anti-cancer, anti-viral and anti-psoriasis treatment.

The following examples further illustrate the invention process. These examples are not intended to limit the invention in any manner. In these examples all temperatures are in degrees Celsius unless otherwise indicated.

#### EXAMPLE I

##### Procedure for the Purification of Refractile Bodies

##### A. Cell Growth

E. coli K-12/MM294-1, which were deposited with the American Type Culture Collection in Rockville, MD under ATCC No. 39,515, and were transformed with recombinant plasmid pBR322 carrying heterologous genes under E. coli trp promoter-operator control, exemplified below, were grown in a 10- or 1000-liter fermentor at 37.degree. C. The dissolved oxygen was maintained at about 40% by, as necessary, (1) increasing agitation; (2) adding air; and (3) adding oxygen. The growth medium was the following:

	Ingredient Concentration
	(NH.sub.4).sub.2 SO.sub.4 72 mM KH.sub.2 PO.sub.4
21 mM MgSO.sub.4.7H.sub.2 O	3 mM Na.sub.3 citrate .2H.sub.2 O
1.5 mM MnSO.sub.4.4H.sub.2 O	30 .mu.M ZnSO.sub.4.7H.sub.2 O
30 .mu.M CuSO.sub.4.5H.sub.2 O	3 .mu.M L-tryptophan
70 mg/L FeSO.sub.4.7H.sub.2 O	72 .mu.M thiamine .HCl
20 mg/L glucose	5 g/L tetracycline
5 mg/L pH	controlled at 6.8 with KOH

A glucose feed was also employed to maintain glucose concentration between 5-10 g/L. The inoculum was 2 mg/L from either frozen or seed cultures. Induction of heterologous protein production by depletion of L-tryptophan from the culture medium occurred at about OD.sub.680 =10 followed by the addition of casamino acids to a final concentration of 2% at OD.sub.680 =15. Cultures were harvested 3-5 hours later.

##### B. Isolation of Heterologous Protein

The general purification scheme for a heterologous protein utilizing refractile body isolation is diagrammed in FIG. 1. The selection of the denaturing agent in solubilizing the desired protein from the inclusion body preparation as well as the additional steps required in the purification process will be dependent upon the nature of the protein and have been described to some extent by Marston, et al.



supra and Kleid, et al., supra. Details of the invention, however, will be similar in all cases and are described below.

#### 1. 10-L Scale

After a 10-L fermentation, cells were concentrated about 10-fold using a hollow fiber membrane cartridge. In addition, cells were washed with 1 L of deionized water. EDTA was added to 25 mM and cells were disrupted by 3 passes at 7500 psi in the homogenizer with brine cooling. The system was rinsed with 0.5 L deionized water and deionized water was added to a final volume of 3 L (Disruptate 1). This lysate was concentrated to 2 L in a housing with a cassette (0.45 micron) followed by diafiltration versus 5 volumes of 5 mM EDTA (diafiltered disruptate). The retentate was concentrated to approximately 1 L and the system was rinsed with 0.5 L deionized water. The concentrated retentate was redispersed by 5 passes at 7500 psi in the homogenizer with brine cooling to ensure complete cell lysis (Disruptate 2).

The homogenizer was rinsed with an equal volume plus 0.1 L 63% sucrose and 2 mM EDTA resulting in about 33-35% final sucrose composition. The final solution density should be between 1.1 and 1.25 g/ml. The temperature and weight of a 10 ml sample of a disruptate was recorded. A temperature of at least 20.degree. C. was maintained prior to centrifugation. The mixture was centrifuged at 12,000.times.g at 75 mls/min.+-.5 mls/min. The supernatant was hazy but not turbid (Supernatant 1).

The supernatant was decanted and the pellet was removed into a beaker and weighed. The pellet was resuspended in 1.5 L 10 mM EDTA with a probe (Resuspension 1) and recentrifuged at the same temperature and flow rate used previously. The supernatant was again decanted (Supernatant 2) and the pellet containing purified refractile body particles (Final Pellet) was stored as frozen paste at -80.degree. C. Refractile particle preparations were characterized by Lowry assay of total protein bioactivity, SDS-PAGE, and lipopolysaccharide assay.

#### 1. 1000-L Scale

The purification scheme for the isolation of refractile bodies from 1000-L of culture is essentially the same as that described for the 10-L scale except for the use of larger scale equipment. Cultures were concentrated by cross-flow filtration using a spiral cartridge. Cells were disrupted by 3 passes through a disruptor at about 6500 psi. After diafiltration versus deionized water, EDTA was added to a final concentration of 2 mM. To ensure that no viable recombinant organisms remained before containment was broken, 1 L of octanol was also added to the fermenter. After several hours, the diafiltered disruptate was again disrupted by one pass through the disruptor.

Sucrose was added to the disruptate to give a final density between 1.1 and 1.25 g/ml. A temperature of at least 20.degree. C. was maintained prior to and during centrifugation. The mixture was centrifuged at 10,000-20,000 .times.g at 1-2 lpm. The resulting pellet containing purified refractile body particles was stored as a frozen paste at -80.degree. C. The refractile body preparation was subsequently characterized by Lowry assay of total protein, bioactivity, SDS-PAGE, and lipopolysaccharide assay.

### EXAMPLE II

#### Purification of Interleukin-2 (IL-2) Containing Refractile Bodies

E. coli K12/MM294-1 cells carrying pLW45 (ATCC No. 39,626) as described in commonly owned copending U.S. Pat. No. 4,530,787, filed Oct. 17, 1984 and issued July 23, 1985 to Z. Shaked et al., were grown as described in Example I(A). Refractile bodies were purified by the method detailed in

Example I(B).

### A. 10-L Scale

The preparation of refractile bodies is characterized at each step of the purification in Table I. Generally, IL-2 protein represents about 50% of the total protein in the purified particles. The other 50% is presumably contributed by E. coli cellular proteins. The Lowry data shown in Table I were some 20% higher than normal due to a low BSA standard, thus resulting in a lower apparent IL-2 contribution to the total particle protein. From densitometry scanning of SDS-PAGE resolution of refractile body protein, IL-2 may represent as much as 85% of the total refractile body protein. This is generally a less accurate means of estimating refractile body protein composition since densitometric absorption depends on the amount of Coomassie dye bound to a protein which varies among different proteins (per unit mass). At any rate, the majority of E. coli proteins found in the refractile bodies are of a much higher molecular mass than the 14.4 kd IL-2 protein and can therefore be removed more easily by alternative methods.

The recovery of IL-2 from cell harvest to the particle paste comprising refractile bodies was about 50% in the example shown in Table I. By washing carefully during the processing, recoveries may reach as high as 70%. These results are compared to the recoveries by the currently used process, which range from 20-25%. Furthermore, a purification of about 7.4-fold was also achieved. Lipopolysaccharide assay results indicate that endotoxin levels in the resuspended particle pellet were about 2-20  $\mu\text{g/ml}$  (or about 40-400  $\text{ng/mg}$ ) protein.

TABLE I

[illegible]

Lowry, O. H. et al. (1951) J. Biol. Chem. 193:265-275. <sup>.sup.(B)</sup> Estimated by densitometry scanning on the basis of IL2 standards run on SDS-PAGE versus samples. <sup>.sup.(C)</sup> Samples were assayed for the presence of IL2 activity by methods described in Gillis, S., et al. (1978) J. Immunol. 120:2027-2032.

### B. Higher Specific Activity IL-2 in the Refractile Body Purification of this Invention

Purification of refractile bodies from the same fermentation by the method described herein as well as by the method described by Marston et al. gave the following results:

TOTAL BIO- SPECIFIC PROTEIN ACTIVITY									
ACTIVITY (MG/ML)		(UNITS/ML)		(UNITS/MG)					
INVENTION	7.1	2.9	.times.	10.sup.5	4.1	.times.	10.sup.5	REFRACTILE BODY PELLE	MARSTON
42.5	2.1	.times.	10.sup.5	5.0	.times.	10.sup.4	REFRACTILE BODY PELLE		

Thus, it is apparent that (1) the additional diafiltration versus deionized water to remove particle associated contaminants and (2) the increased density due to sucrose addition to keep material less dense than refractile bodies from pelleting result in a specific activity of IL-2 over eight times higher than obtained with the previous method.

### C. 1000-L Scale

The preparation of refractile bodies is characterized at several steps of the purification in Table II, which represents a first run. Again as in the case of the 10-L growth, IL-2 represents approximately 50% (53.5%) of the total refractile body protein. The final specific activity is slightly lower than, but within experimental variability of, that obtained in the 10-L preparation. Table III shows the recovery of IL-2 from the harvest to the final refractile body pellet obtained after centrifugation of the sucrose suspension at 10,000-20,000.times.g. Some 11.3% of the initial IL-2 protein as estimated by densitometry scanning of SDS-PAGE separated proteins was recovered while about 25% of the initial IL-2 bioactivity was recovered. In the second run indicated in Tables IV and V and in FIG. 3, the IL-2 represented approximately 50% (45.7%) of the total refractile body protein, and about 17-23% of the initial IL-2 bioactivity and protein, respectively, was recovered.

SDS-PAGE (15%, reducing) analysis of the material at each step of refractile body purification is shown in FIG. 3. Lane A of the SDS-PAGE contains molecular mass markers of 94, 67, 43, 30, 20, and 14.4 kilodaltons from top to bottom. Lanes B-E each contain 20 .mu.g of protein from the cell culture harvest, the concentrated culture, the disruptate, and the diafiltrate, respectively. Lanes F-H each contain 15 .mu.g of protein showing the two supernatant fractions from the first centrifugation (5 .mu.g each of the first refractile body pellet are in lanes I and J), and the supernatant from the second centrifugation (5 .mu.g of the second refractile body pellet are shown in lane K). Lanes L, M, and N contain 3.6, 1.8, and 0.9 .mu.g, respectively, of purified IL-2 as a standard. The SDS-PAGE indicates that by Lanes I and J representing the first refractile body pellet, the IL-2 is markedly more free of high molecular weight contaminants.

The percentages for recovery indicate a somewhat lower yield of IL-2 in the scaled-up procedure, which is not unexpected due to extensive apparatus involved in processing such large volumes. Recoveries would be expected to improve upon further refinement of the procedure. For example, preliminary results obtained from the fermentation and purification run described in Tables IV and V by the SDS-PAGE suggest that a second centrifugation of the sucrose supernatant yielded pellets with additional refractile bodies. As much as 50-80% of the first pellet mass may be obtained by additional centrifugation. Initial characterizations of this material indicated that it had a purity comparable to that of the first pellet (see FIG. 3, lanes J and K). The figure shows that the IL-2 appears to be quite pure in the disruptate pellet. Estimates of recovery of IL-2 may be as high as about 50%. The majority of the contaminating E. coli proteins are of much higher molecular weight, thereby facilitating further purification of the desired product. The cost reduction and ease in handling realized with the refractile body purification process herein compared with the previously utilized process further enhances its value.

TABLE II

1000 L SCALE (FIRST RUN) SPECIFIC TOTAL PROTEIN % IL-2 BY IL-2 BIOACTIVITY											
ACTIVITY SAMPLE (MG/ML).sup.(A) DENSITOMETER SCAN (MG/ML).sup.(B)											
(UNITS/ML).sup.(C) (U/MG											
											PROTEIN)
HARVEST	11.27	12.9	1.5	9.9	.times.	10.sup.5	8.8	.times.	10.sup.4	CONCENTRATE	32.87
	4.9	.times.	10.sup.6	1.5	.times.	10.sup.5	DISRUPTATE	16.36	16.9	2.8	4.8
								.times.	10.sup.6	2.9	.times.

10.sup.5 SUCROSE 10.14 13.6 1.4 1.4 .times. 10.sup.6 1.4 .times. 10.sup.5 SUPERNATANT  
SUCROSE PELLET 10.27 53.5 5.5 9.4 .times. 10.sup.6 9.2

.times.  
10.sup.5 .sup.(A) Lowry, O. H. et. al. (1951) J. Biol. Chem. 193:265-275. .sup.(B) Estimated by  
densitometry scanning on the basis of IL2 standards run on SDSPAGE versus samples. .sup.(C) Samples  
were assayed for the presence of IL2 activity by methods described in Gillis, S., et al. (1978) J.  
Immunol. 120:2027-2032.

TABLE III

1000 L SCALE RECOVERY (FIRST RUN)	RECOVERY	TOTAL	TOTAL IL-2	TOTAL BIOACTIVITY (% BY WEIGHT- FOLD SAMPLE PROTEIN (G) PROTEIN (G) (UNITS) (% BY ACTIVITY) PURIFICATION	HARVEST
11,300 1500 9.9 .times. 10.sup.11 100 1 PARTICLE REFRACTILE 266 169.2 2.5 .times. 10.sup.11					
11.3-25 4.8 BODY PELLET					

TABLE IV

1000 L SCALE (SECOND RUN) TOTAL PROTEIN % IL-2 BY IL-2 BIOACTIVITY SPECIFIC ACTIVITY SAMPLE (MG/ML).sup.(A) DENSITOMETER SCAN (MG/ML).sup.(B) (UNITS/ML).sup.(C) (UNITS/MG	IL-2)
HARVEST 9.1 12.5 1.14 1.7 .times. 10.sup.6 1.9 .times. 10.sup.5 CONCENTRATE 29.3 15.5 4.54 4.9 .times. 10.sup.6 1.7 .times. 10.sup.5 DISRUPTATE 26.5 15.0 3.98 5.6 .times. 10.sup.6 2.1 .times. 10.sup.5 DIAFILTRATE 13.8 19.2 2.65 2.6 .times. 10.sup.6 1.9 .times. 10.sup.5 SUCROSE 6.0 12.8 0.77 1 .times. 10.sup.6 1.7 .times. 10.sup.5 SUPERNATANT SUCROSE PELLET 3.0 45.7 1.38 1.5 .times. 10.sup.6 5.0 .times. 10.sup.5	

.sup.(A)  
Lowry, O. H. et al. (1951) J. Biol. Chem. 193:265-275. .sup.(B) Estimated by densitometry scanning on  
the basis of IL2 standards run on SDSPAGE versus samples. .sup.(C) Samples were assayed for the  
presence of IL2 activity by methods described in Gillis, S., et al. (1978) J. Immunol. 120:2027-2032.

TABLE V

1000 L SCALE RECOVERY (SECOND RUN) TOTAL TOTAL IL-2 TOTAL BIOACTIVITY RECOVERY FOLD SAMPLE PROTEIN (G) PROTEIN (G) (UNITS/ML) (% BY WEIGHT) PURIFICATION	9
HARVEST 9,090 1140 1.7 .times. 10.sup.12 100 1 PARTICLE REFRACTILE 577 264 2.9 .times. 10.sup.11 17-23 3.8 BODY PELLET	

## EXAMPLE III

## Formulation of Purified Interleukin-2 (IL-2)

Following the isolation of the particle paste containing refractile bodies described in Example II.C., the paste was further processed to obtain highly purified IL-2 protein. Initially, the paste was solubilized in phosphate buffered saline containing 5% SDS. The solubilized material was centrifuged at 25,000-35,000.times.g to remove insoluble materials. The supernatant from the centrifugation was reduced by the addition of solid DTT to a final concentration of 50 mM and of EDTA to 2 mM. The pH of the solution was adjusted to 8.5.+-.0.1 with NaOH and then heated to 50.+-.5.degree. C. for 20 minutes



under nitrogen. Following the reduction, the reaction was cooled to about 25.degree. C. and the pH was readjusted to 5.5. $\pm$ .0.1 using glacial acetic acid.

Chromatographic separation of the higher molecular weight contaminants was achieved next using a Sephacryl.RTM.S-200 column. The solubilized and reduced refractile body protein was loaded onto the column and fractions were collected into clean, depyrogenated vessels using an elution buffer containing 50 mM acetate pH 5.5, 1 mM EDTA and 0.1% SDS. Peak fractions (those falling within 70% of the maximum peak height) were pooled and subjected to a controlled oxidation as follows: The S-200 protein pool and iodosobenzoic acid, in a molar ratio of 1:1.6, respectively, were added to a reaction vessel containing 10 mM sodium phosphate, 0.1% SDS and 1 mM EDTA. The pH was controlled at 7.8. $\pm$ .0.2 with 0.5N NaOH during oxidation and adjusted to 5.5. $\pm$ .0.2 when oxidation was completed. Since oxidized IL-2 is more hydrophilic than reduced IL-2, the progress of the oxidation reaction was monitored by RP-HPLC.

Oxidized IL-2 was concentrated using a hollow fiber ultrafiltration unit with a 10,000 molecular weight cutoff. The protein was then diafiltered against 0.1% SDS, 50 mM acetate pH 5.5 and 1 mM EDTA for three volume exchanges. In preparation for the subsequent HPLC purification, the pH of the diafiltered protein was lowered to 3 or less using glacial acetic acid and filtered through a 0.45  $\mu$ m filter.

Preparative HPLC using a Vydac C.sub.4 bonded phase silica gel column supplied with two solvents was the next step in the IL-2 purification scheme. Solvent 1 was 6% acetic acid and 10% 2-propanol in distilled water, and solvent 2 was 6% acetic acid and 94% 2-propanol in distilled water. After pumping solvent 1 for 30 minutes, the acidified IL-2 protein was loaded. The column was developed with a gradient of solvents 1 and 2 and the protein which eluted at about 40% solvent 2 was pooled into a depyrogenated graduated cylinder. Pooled protein was diluted by slowly adding it to a stirred buffer solution containing 50 mM acetate pH 5.5, 1 mM EDTA and 0.1% SDS that had 14 times the volume of the HPLC pool. Dilution was required due to the sensitivity of the hollow-fiber ultrafiltration unit used for concentration in the next step to organic solvents present in the HPLC pool.

The diluted HPLC pool was concentrated using a hollow-fiber ultrafiltration unit with a 10,000 molecular weight cutoff. The concentrate was diafiltered against 50 mM acetate pH 5.5, 1 mM EDTA and 0.1% SDS with three volume exchanges.

The final chromatographic step in the purification of IL-2 involved a second Sephacryl.RTM.S-200 column. The primary objective of this column was to separate the IL-2 monomer fractions from higher molecular weight oligomers of the protein. The column was eluted with buffer containing 50 mM acetate pH 5.5, 1 mM EDTA and 0.1% SDS, and IL-2 monomer fractions were pooled. Immediately preceding formulation, the protein was diafiltered against 10 mM sodium phosphate pH 7.5 until the SDS level was in the range of 100-200  $\mu$ g/mg protein.

Purified IL-2 was formulated in 10 mM sodium phosphate pH 7.5 with 5% mannitol (w/v). It was prefiltered through a 0.45  $\mu$ m filter and sterile filtered through a 0.22  $\mu$ m filter. Finally, the product was lyophilized to dryness in the container vial for storage at 4.degree. C. The purified and formulated IL-2 protein produced in this manner was found to be 97% pure by HPLC and 99% pure IL-2 monomer by either reduced or non-reduced SDS-PAGE. The specific activity was 2.3.times.10<sup>sup.6</sup> units/mg protein and the level of residual SDS was 181  $\mu$ g SDS/mg IL-2, 100-200  $\mu$ g SDS/mg IL-2 being required to maintain the desired IL-2 solubility. The amino-terminal amino acid sequence and the amino acid composition of the final product agreed with theoretical predictions.

#### EXAMPLE IV

## Purification of Human Fibroblast Interferon (IFN-.beta.) Containing Refractile Bodies

E. coli K12/MM294-1 cells carrying pSY2501 (ATTC No. 39,517) as described in U.S. Pat. No. 4,518,584, assigned to Cetus Corporation, were grown as described in Example 1(A).

## A. Purification of Refractile Bodies

Refractile bodies were purified by the method detailed in Example I(B)(1) with some modifications. Cells were disrupted in the homogenizer by three passes at 7500 psi. The system was rinsed with deionized water and the lysate was brought to a final volume of 5 L with deionized water (Disruptate 1). Diafiltration versus five volumes of deionized water (diafiltered disruptate) was followed by concentration of the cell particle material and a rinse of the system with 0.75 L 10 mM EDTA. The final volume of this diafiltered cellular concentrate was 2.1 L. The concentrate was redispersed by three passes at 7500 psi in the homogenizer, and an equal volume of 63% sucrose and 2 mM EDTA was added to give a volume of 5.0 L (Disruptate 2). The mixture was centrifuged at 40,000 rpm in a Sharples centrifuge at about 100 ml/min. The system was rinsed with 1.0 L 10 mM EDTA. The final pellet containing purified refractile body particles (Final Pellet) was stored as a frozen paste at -80.degree. C.

## B. Characterization of Refractile Body Preparation

The preparation of refractile bodies is characterized at each step of the purification in Table VI. It appears that IFN-.beta.

TABLE VI

10 L SCALE SPECIFIC TOTAL ACTIVITY TOTAL VOL- PROTEIN IFN-.beta. BIOACTIVITY (UNITS/MG IFN.beta. RECOVERY FOLD SAMPLE UME (MG/ML).sup.(A) (MG/ML).sup.(B) (UNITS/ML).sup.(C) IFN-.beta.) (g) (% BY WEIGHT) PURIF													
													HARVEST
10.64 L	5.4	0.19	--	--	2.02	--	--	CONCENTRATE	3.15 L	19.5	0.68	2.4	.times. 10.sup.8 1.2 .times.
10.sup.7	2.14	100	1.0	DISRUPTATE	1	3.78 L	13.8	0.62	1.8	.times. 10.sup.8 1.3 .times. 10.sup.7 2.34			
109	1.28	DIAFILTRATE	28.0 L	0.5	N.D.	1.2	.times. 10.sup.7 2.4 .times. 10.sup.7 N.D.	0	N.A.				
DIAFILTERED	2.1 L	16.4	1.02	3.2	.times. 10.sup.8 2.0 .times. 10.sup.7 2.14	100	1.8	CELLULAR					
CONCENTRATE	DISRUPTATE	2	5.0 L	6.7	0.44	1.3	.times. 10.sup.8 1.9 .times. 10.sup.7 2.20	102	1.9				
SUPERNATANT	5.8 L	4.2	0.08	4.2	.times. 10.sup.7 1.0 .times. 10.sup.7 0.46	22	PARTICLE	55.3 g					
8.97 g	1.54	.sup. 7.3 .times. 10.sup.11	8.1	.times. 10.sup.7 1.71	85	4.9	PELLET						

.sup.(A)  
Lowry, O. H. et al. (1951) J. Biol. Chem. 193:265-275. .sup.(B) Estimated by densitometry scanning of IFN.beta. standards run on SDS-PAGE versus samples. .sup.(C) Samples were assayed for the presence of IFN.beta. antiviral activity by methods described in W. E. Stewart, "The Interferon System", Springer-Verlag, p17-18 (1979). N.D. = not detectable N.A. = not applicable

represented about 17% of the total protein in the purified particles. The other 83% was presumably contributed by E. coli cellular proteins. The recovery of IFN-.beta. from cell harvest to the particle paste comprising refractile bodies was 85%, indicating that almost all of the IFN-.beta. produced by E. coli carrying pSY2501 was contained in refractile bodies. The additional contents of these refractile bodies limited the degree of purification of IFN-.beta. which could be achieved by simple isolation of refractile bodies. However, most contaminants are of a much higher molecular mass and can therefore be removed more easily by alternative methods. Furthermore, this simple, high-recovery "front end" process for recovering recombinant IFN-.beta. does not require the use of an aliphatic alcohol to extract this lipophilic protein from the aqueous medium in which it is produced. The next example illustrates the further developed high-recovery "front end" process wherein an organic extraction is employed.



## EXAMPLE V

## Purification of Human Fibroblast Interferon (IFN-.beta.) Containing Refractile Bodies

This example delineates the alternative expanded front-end process for recovery of the final pellet containing refractile bodies having approximately 80% (81.4%) IFN-.beta.. E. coli K12/MM294-1 cells carrying pSY2501 (ATCC No. 39,517) as described in U.S. Pat. No. 4,518,584, assigned to Cetus Corporation, were grown essentially as described in Example 1(A) for a 1000-liter fermentation run. The differences between Example 1(A) and that for cell growth in this example were the following:

1. Once the fermenter was filled with water to the operating volume, the following trace elements were added:

(i)  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$

$\text{MnSO}_4 \cdot \text{H}_2\text{O}$

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

(ii)  $\text{Na}_3\text{Citrate} \cdot 2\text{H}_2\text{O}$

(iii)  $\text{KH}_2\text{PO}_4$

(iv)  $(\text{NH}_4)_2\text{SO}_4$  ;

2. the fermenter feed and addition vessels were then sterilized according to standard operating procedures;

3. the fermenter was cooled and inoculated with frozen or seed E. coli culture;

4. no tetracycline was added to the fermentation broth;

5. 100  $\mu\text{M}$ , rather than, 72  $\mu\text{M}$   $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was used;

6. 20 mM, rather than 3 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  was used;

7. at approximately 15 hours after fermentation was begun, the pH was adjusted to 6.8;

8. optical density measurements and residual glucose measurements on samples were taken at 14-16 hours and approximately 1 hour intervals thereafter; and

9. the cultures were harvested when glucose consumption reached 40. $\pm$ .6 g/l.

## EXPANDED FRONT-END PROCESS

The general purification scheme for a heterologous protein utilizing the expanded front-end process for the isolation of refractile bodies is diagrammed in FIG. 2. As indicated in Example I(B), supra, the selection of the denaturing agent for solubilizing the desired protein from the inclusion body preparation as well as the additional steps required in the purification process will be dependent upon the nature of the protein and have been described to some extent by Marston et al., supra, and Kleid et al., supra.

At various steps of the expanded front-end process used in this example, the refractile body preparations were characterized by Lowry assay of total protein bioactivity, SDS-PAGE and lipopolysaccharide assay. Table VII characterizes preparation of refractile bodies at various steps of the purification up to the final pellet. From the densitometer scan of SDS-PAGE results, it appears that IFN-.beta. represented about 81.4% of the total protein in the final pellet.

Cultures were concentrated approximately 5-10 fold by circulating the harvest material (Harvest) under pressure through UF cross-flow filtration cartridges with a 100K molecular weight cutoff (Concentrate). Cells were disrupted by 3 passes through a Manton-Gaulin high-pressure homogenizer at 6,000 to 8,000 psi (Disruptate I).

EDTA was added to the disruptate to a final concentration of 5 mM. The suspension was then diafiltered against 5 volumes of deionized water (diafiltered disruptate).

EDTA was then added to a final concentration of 2 mM. Octanol was added to 1% (v/v) to kill any residual live bacteria in the diafiltered product. The suspension was redisrupted by passing it twice through the Manton-Gaulin high-pressure homogenizer at 6,000-8,000 psi (Disruptate II).

Sucrose was added to the redisruptate to a final concentration of 23% (wt/wt), creating a final density gradient between 1.1 and 1.25 g/ml (Sucrose Suspension). The mixture was centrifuged at 10,000 to 15,000 xg, and the particle pellet or paste was collected (Particle Pellet). As indicated by densitometer scan the particle pellet contained approximately 20.4% IFN-.beta..

The particle pellet was then solubilized in phosphate buffered saline with 2% SDS. Solid DTT and EDTA were added to a final concentration of 10 mM and 2 mM, respectively. The suspension was heated to 50.degree..+-.5.degree. C. for 10 minutes under nitrogen. The reaction mixture was then cooled to approximately 25.degree. C., and then the pH of the mixture was adjusted to 7.4.

A volume of 2-butanol equal to the total volume of the suspension was measured. The suspension and organic solution were pumped separately but simultaneously at flow rates of 1.1 to 1.3 liters per minute through a static mixer and then into a continuous centrifuge (Westfalia at approximately 11,770 xg) for phase separation. The 2-butanol-rich phase containing the IFN-.beta. was collected (Organic Extract).

The 2-butanol extract was mixed with 2.5 volumes of 0.1% SDS in phosphate-buffered saline. Solid DTT was added to a final concentration of 2 mM. The pH of the organic extract/buffer solutions was adjusted to 6.2+-.0.1 with glacial acetic acid (Acid Precipitate).

The mixture was then centrifuged (Sharples centrifuge at 13,200 xg) for approximately 2-6 hours. The final pellet was then collected (Final Pellet) containing approximately 81% IFN-.beta..

## DOWNSTREAM PROCESSING

The final pellet was then re-suspended with 5% SDS in 50 mM phosphate buffer and 5 mM EDTA. Solid DTT was added to a final concentration of 20 mM, and the pH was adjusted to 8.5 with NaOH. The suspension was heated to 50.degree..+-.5.degree. C. for 10 minutes under nitrogen, and then cooled to approximately 25.degree. C. The pH was then adjusted to a pH of 5.5 with glacial acetic acid, and the solution was filtered through a 0.65 .mu.m filter.

The filtrate was then processed by pre-column chromatography by loading a Sephacryl.RTM. S200 column and collecting fractions into clean, depyrogenated vessels using an elution buffer that is

composed of 50 mM acetate, pH 5.5, 1 mM EDTA and 1% SDS. The fractions containing the IFN-.beta. monomer were pooled.

The pre-column pool was then concentrated by using a hollow-fiber ultrafiltration unit with a 10K molecular weight cut-off.

The concentrated pre-column pool was then oxidized using iodosobenzoic acid (IBA). The oxidation was effected by adding equimolar amounts of protein and IBA into a reaction vessel containing 2 mM sodium pyrophosphate, 0.1% SDS and 1 mM EDTA. A 20 .mu.M excess of IBA was present at the end of the oxidation. The pH was controlled at 9.0+-.0.1 with NaOH during oxidation, and adjusted to 5.5+-.0.2 with glacial acetic acid when the oxidation was completed.

The protein was then concentrated using a hollow-fiber ultrafiltration unit with a 10K molecular weight cut-off.

The protein was then loaded onto the main column (Sephacryl.RTM. S200-A), and fractions were collected into clean, depyrogenated vessels using an elution buffer that is composed of 50 mM acetate, pH 5.5, 1 mM EDTA and 0.1% SDS.

A SDS-PAGE was performed on samples from each fraction tube starting from the beginning of the peak to be pooled to the end of the peak. Using the SDS-PAGE results, the fractions containing no high molecular weight contaminants were determined. Those fractions were then pooled.

The main column pool was then concentrated by using a hollow-fiber ultrafiltration unit with a 10K molecular weight cut-off.

The above procedure performed with the main column was repeated on a Sephadex.RTM. G-75 column. Using the SDS-PAGE results, the fractions containing neither low nor high molecular weight contaminants were pooled.

A Sephadex.RTM. G-25 column was then equilibrated with 1 mM NaOH and loaded with the Sephadex.RTM. G-75 pool. Using the process chromatogram, the IFN-.beta. peak was collected. The product was formulated within 15 minutes from this desalting process.

The purified IFN-.beta. was formulated with Normal Serum Albumin (Human) USP (NSA) and 50% Dextrose Monohydrate. Normal Serum Albumin was diluted with water for injection to give a final concentration of 1.25% for 0.05 and 0.25 mg/ml IFN-.beta. formulations or of 5.0% for a 1.00 mg/ml IFN-.beta. formulation. The pH of the diluted NSA solution was adjusted to 12.0+-.0.5 with 10% NaOH.

The IFN-.beta. was immediately added to the NSA solution, and the pH of the mixture was adjusted to 7.5+-.0.3 with 3-6N HCl. The calculated amount of dextrose was then added.

The formulated product was pre-filtered through a 0.45 .mu.m filter and then filtered through a sterile 0.22 .mu.m filter within 4 hours.

Then sterilized vials with sterilized stoppers and components were aseptically filled with the IFN-.beta. formulations under sanitary and sterile conditions that were environmentally monitored.

The vials were placed in a lyophilizer where appropriate thermocouples were attached. The vials were frozen to between -35.degree. and -45.degree. C. The lyophilization cycle was completed and the vials

were mechanically sealed under a vacuum.

TABLE VII

SCALE SPECIFIC ACTIVITY	TOTAL PROTEIN	IFN-.beta.	BIOACTIVITY (UNITS/MG	% IFN-
.beta. BY SAMPLE (MG/ML).	sup.(A) (MG/ML).	sup.(B) (UNITS/ML).	sup.(C) IFN-.beta.)	DENSITOMETER
HARVEST 7.31	0.32	3.3 .times. 10.sup.6	1.0 .times. 10.sup.7	6.6 CONCENTRATE 24.56
1.8 .times. 10.sup.7	1.4 .times. 10.sup.7	6.4 DISRUPTATE I	15.65	1.10 5.6 .times. 10.sup.6
5.1 .times. 10.sup.6	6.3 DIAFILTRATE 11.53	0.81 8.9 .times. 10.sup.6	1.1 .times. 10.sup.7	6.6 DISRUPTATE II
11.09 0.66 5.0 .times. 10.sup.6	7.6 .times. 10.sup.6	6.4 SUCROSE SUSPENSION	11.42	0.76 8.9 .times. 10.sup.6
1.2 .times. 10.sup.7	6.8 PARTICLE PELLET 4.07	0.64 1.5 .times. 10.sup.7	2.3 .times. 10.sup.7	20.4 ORGANIC EXTRACT 0.57
0.21 6.3 .times. 10.sup.6	3.0 .times. 10.sup.7	88.3 ACID PRECIPITATE 0.18	0.05 1.8 .times. 10.sup.6	3.6 .times. 10.sup.7
80.8 FINAL PELLET 2.68	2.12 5.6 .times. 10.sup.7	2.7 .times. 10.sup.7	81.4	

Lowry, O. H. et al. (1951) J. Biol. Chem. 193:265-275. .sup.(B) Estimated by densitometry scanning on the basis of IFN.beta. standards run on SDSPAGE versus samples. .sup.(C) Samples were assayed for the presence of IFN.beta. antiviral activity by methods described in W. E. Stewart, "The Interferon System," SpringerVerlag, pp. 17 & 18 (1979).

In summary, it can be seen that the present invention provides an efficient process for isolating refractile bodies containing heterologous proteins from the host disruptate in which the refractile bodies are contained. In the process herein, cost reduction and ease in handling are realized.

Modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of pharmaceutical formulation or related fields are intended to be within the scope of the following claims.

\* \* \* \* \*

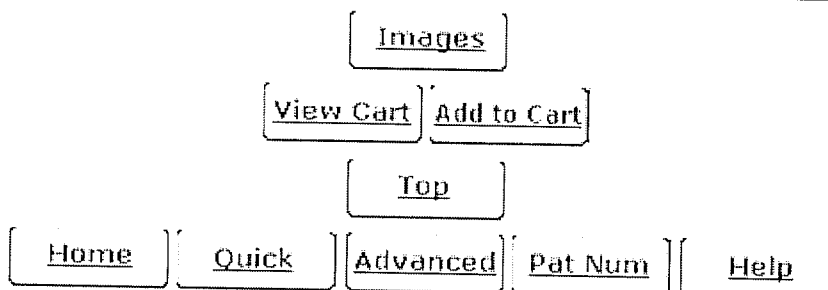


EXHIBIT O  
PATENT 4,572,798

USPTO PATENT FULL-TEXT AND IMAGE DATABASE

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( 1 of 1 )

**United States Patent**  
**Koths , et al.**

**4,572,798**  
**February 25, 1986**

Method for promoting disulfide bond formation in recombinant proteins

**Abstract**

Reduced cysteine-containing proteins consisting of recombinant IFN-.beta., IL-2 or muteins thereof may be oxidized selectively so that the recombinant proteins have essentially the same disulfide bridging and biological activity as their native counterparts. The oxidized product is substantially free of unwanted side products and contains a minimal amount of intermolecular oligomers. The oxidation takes place in an aqueous medium containing a solubilizing agent at a pH of about 5.5 to 9, preferably at a pH of about 7. The reaction is initiated by addition of at least an effective amount of an oxidation promoter containing a Cu.sup.+2 cation such as CuCl.sub.2 or o-phenanthroline/Cu.sup.+2 complex in the presence of air.

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**Current International Class:** C07K 1/00 (20060101); C07K 1/113 (20060101); C07K 14/435 (20060101); C07K 14/565 (20060101); C07K 14/55 (20060101); C07G 007/00 (); A61K 045/02 ()

**Field of Search:** 260/112R 424/85 435/811

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114507

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*Attorney, Agent or Firm:* Halluin; Albert P. Hasak; Janet E.

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**Claims**


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What is claimed is:

1. A method of oxidizing a fully reduced recombinant protein selected from the group consisting of interferon-beta, interleukin-2 and muteins thereof, whereby cysteines are oxidized preferentially to form the disulfide bridges which correspond to those present in the naturally occurring protein, which method comprises reacting an aqueous solution containing a solubilized form of the recombinant protein at a pH between about 5.5 and 9 in the presence of air with at least an effective amount of an oxidation promoter containing a Cu.sup.+2 cation.
2. The method of claim 1 wherein the recombinant protein is a mutein of said protein having at least one of its cysteine residues which is free to form a disulfide bond and is nonessential to the biological activity of the protein deleted or replaced by another amino acid.
3. The method of claim 2 wherein the mutein is des-ala IL-2.sub.ser125.
4. The method of claim 2 wherein the mutein is IFN-.beta..sub.ser17.
5. The method of claim 1 wherein the pH is between about 6 and about 8.
6. The method of claim 1 wherein the pH is about 7.
7. The method of claim 1 wherein the oxidation promoter is CuCl.sub.2 or (o-phenanthroline).sub.2 Cu.sup.+2.
8. The method of claim 1 wherein the oxidation promoter is CuCl.sub.2.
9. The method of claim 1 wherein the oxidation promoter is (o-phenanthroline).sub.2 Cu.sup.+2.
10. The method of claim 1 wherein the concentration of said protein is in the range of about 0.05 to

about 2 mg/ml.

11. The method of claim 1 wherein the concentration of said oxidation promoter is approximately equivalent to the concentration of free sulfhydryl groups on the protein which are intended to be oxidized to form cystines.

12. The method of claim 10 wherein the oxidation promoter is CuCl<sub>2</sub> and its concentration ranges from about 1 to 400 micromolar.

13. The method of claim 1 wherein the reaction is carried out at a temperature of from 20.degree. to 40.degree. C.

14. The method of claim 1 wherein the protein is solubilized with sodium dodecyl sulfate.

15. The method of claim 14 wherein the concentration of sodium dodecyl sulfate prior to reaction ranges from about 0.05% to 2% by weight per volume.

16. The method of claim 1 wherein the protein is an IL-2 or IFN- $\beta$  mutein, the oxidation promoter is CuCl<sub>2</sub> in an amount of from about 5 to 50 micromolar, the pH of the reaction is about 7, the concentration of IL-2 in the reaction mixture ranges from about 0.1 to about 1 mg/ml, and sodium dodecyl sulfate is present as a solubilizing agent at a concentration ranging from about 0.1 to 1% by weight per volume.

17. The method of claim 16 wherein the mutein is des-ala IL-2.sub.ser125.

18. The method of claim 16 wherein the mutein contains the three cysteines present in native IL-2.

19. The method of claim 16 wherein the mutein is IFN- $\beta$ .sub.ser17.

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### *Description*

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## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

This invention relates to a method of catalyzing disulfide bond formation in fully reduced, cloned gene products produced in microbes such as Escherichia coli. More particularly, the invention concerns such a method of oxidation wherein the reaction is controlled to promote the in vitro formation of disulfide bridges which correspond to those present in the naturally occurring protein species.

### 2. Description of Related Disclosures

When native proteins which contain one or more disulfide bridges in their native state are produced as recombinant proteins in microorganisms, the protein produced is often in a reduced form, lacking disulfide bridges. In some cases the protein product may contain oligomers following purification. Such oligomers may be the result of uncontrolled oxidation or thiol-disulfide exchange reactions. If the native protein contains disulfide bonds, it will often be desirable to promote chemically the formation of the corresponding disulfide bonds in the recombinant protein product, while minimizing the formation of oligomers or other modified protein by-products. Oxidizing the protein in an uncontrolled manner may

also result in the formation of undesirable isomers (incorrect intramolecular bridging). Such unwanted reactions may complicate the purification of the protein from the culture, reduce the yield of protein having the desired structure, or generate a protein with less than full bioactivity. In the case of certain proteins which are intended for therapeutic use, uncontrolled disulfide bond formation during purification or formulation may yield a nonhomogeneous material which is contaminated with isomers and/or oligomers which may be inactive and/or have increased immunogenicity.

U.S. Pat. No. 4,530,787 issued July 23, 1985 to Ze'ev Shaked et al., entitled "Controlled Oxidation of Microbially Produced Cysteine-Containing Proteins," describes a process for oxidizing such microbially produced proteins in a selective, controlled manner using a non-catalytic oxidizing agent, preferably o-iodosobenzoic acid, which oxidizes cysteines preferentially producing the desired disulfide bridging in high yield. This process requires at least stoichiometric amounts of oxidizing agent to protein to ensure that the oxidation proceeds to completion.

Similarly, a process has been described for the catalysis of disulfide bond formation in microbially produced rennet, using a mixture of oxidized and reduced glutathione in urea. (European patent application No. 83307841.3 published as European Publication No. 114,507 on Aug. 1, 1984 to Hayenga et al.)

It is known that ferricyanide or copper +2 ions are able to catalyze disulfide bond formation in .beta.-lactoglobulin in the presence of sodium dodecyl sulfate. Leslie, J. et al., Can. Jour. Biochem., 46, 625 (1968). Other disclosures teach use of specific divalent metal salts as oxidants for cysteine or the sulfhydryl groups in specific cases: (a) copper ion: (for free cysteine) Hanaki, A. et al., Bull Chem. Soc. Jpn., 56, 2065 (1983); (for sulfhydryls in lysozyme) Yutani, K. et al., J. Biochem., 64, 449 (1968); (for sulfhydryl compounds such as glutathione, cysteine, 2-mercaptoethanol, thioglycolic acid and reduced lipoic acid) Kobashi, K., Biochim. Biophys. Acta, 158, 239 (1968); (b) transition metals: (for cysteine and other mercaptans and proteins with free sulfhydryl groups) Friedman, Mendel, The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins, (New York: Pergamon Press), Chapter 2, pp. 25-50 (1973); and (c) possibly calcium ion: (for sulfhydryls in deoxyribonuclease) Price, P. et al., J. Biol. Chem., 244, 929 (1969).

The mechanism of oxidation in these reactions is unclear, but has been postulated to be based on reactions involving peroxide or free radicals. However, it appears that the ability to predict that a given divalent salt will successfully promote the correct oxidation of a specific protein without extensive side reactions is not possible at this time. The present invention demonstrates the ability of certain metal-containing compounds to promote highly selective and useful formation of disulfides in various forms of recombinant interleukin-2 and .beta.-interferon.

## SUMMARY OF THE INVENTION

The present invention relates to a method of oxidizing a fully reduced recombinant protein selected from the group consisting of interferon-beta, interleukin-2 and muteins thereof, whereby cysteines are oxidized preferentially to form the disulfide bridges which correspond to those present in the naturally occurring protein. This method comprises reacting an aqueous solution containing a solubilized form of the recombinant protein at a pH of between about 5.5 and 9 in the presence of air with at least an effective amount of an oxidation promoter containing a Cu.sup.+2 cation.

The method of this invention minimizes the difficulties encountered during oxidation of specific proteins, including oxidative side reactions, inability to regain full bioactivity, and unwanted oligomer or isomer formation. In addition, the preferred method herein described, using cuprous chloride as an oxidation promoter, has the added advantages of being extremely rapid and involving a reagent which is

easily assayable in and easily removed from the final product. The reaction herein described is active at catalytic as well as stoichiometric concentrations (relative to free sulfhydryls). Thus, the need to monitor the mole ratio of oxidation promoter to protein (in order to achieve 100% disulfide formation) may not be as great as with other oxidation agents. Disulfide bond formation in recombinant interleukin-2 (rIL-2) or .beta.-interferon (rIFN-.beta.) at mg/ml concentrations can be driven to completion in one hour at CuCl.sub.2 concentrations of less than 100 .mu.M.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 represents five reverse-phase high pressure liquid chromatography (RP-HPLC) absorbance profiles of a reaction mixture in which a recombinant mutein of IFN-.beta., having its cysteine residue at position 17 replaced by a serine residue (designated herein as IFN-.beta..sub.ser17), has been oxidized using 8 .mu.M CuCl.sub.2 as the oxidation promoter. FIG. 1A represents a control reaction involving reduced IFN-.beta..sub.ser17 which has been placed in a buffer for 7 minutes without CuCl.sub.2. FIG. 1B represents 7 minutes of oxidation with CuCl.sub.2 ; FIG. 1C represents 14 minutes of oxidation; FIG. 1D represents 28 minutes of oxidation; and FIG. 1E represents 75 minutes of oxidation. FIG. 1F represents a plot of the percent IFN-.beta..sub.ser17 oxidized versus number of minutes of oxidation, based on the RP-HPLC analysis.

FIG. 2 provides a comparison of recombinant IFN-.beta..sub.ser17 after oxidation for 75 minutes in 8 .mu.M CuCl.sub.2 (FIG. 2A) with an aliquot of the same material reduced for 15 minutes in 10 mM dithiothreitol at 50.degree. C. (FIG. 2B).

FIG. 3 represents four RP-HPLC absorbance profiles of a reaction mixture in which a recombinant mutein of IL-2, having the cysteine residue at position 125 replaced by a serine residue and having the N-terminal alanine deleted (designated herein as des-ala IL-2.sub.ser125), has been oxidized using 50 .mu.M CuCl.sub.2 as the oxidation promoter. FIG. 3A represents 2 minutes of oxidation; FIG. 3B represents 10 minutes of oxidation; FIG. 3C represents 50 minutes of oxidation; and FIG. 3D represents a chromatogram of the reaction mixture after the oxidized product of 50 minutes is re-reduced using 10 mM dithiothreitol for 15 minutes at 60.degree. C.

FIG. 4 represents a silver-stained, non-reducing SDS-PAGE analysis of the des-ala IL-2.sub.ser125 protein after 40 minutes of oxidation using 50 .mu.M CuCl.sub.2 as the oxidation promoter to determine the extent of formation of intermolecular sulfhydryl groups (oligomers).

FIG. 5 represents plots of the percent oxidation of the des-ala IL-2.sub.ser125 (as measured by HPLC peak height, less background) versus time in minutes at 25.degree. C. for three different CuCl.sub.2 concentrations. A control reaction containing 10 mM ethylenediamine tetraacetic acid (EDTA) was also run.

FIG. 6 represents a plot of the percent oxidation of the des-ala IL-2.sub.ser125 (as measured by HPLC peak height, less background) versus the pH of the oxidation reaction using 8 .mu.M CuCl.sub.2. The plot shows the effect of varying pH on the reaction rate measured at the point in the reaction at which IL-2 is approximately 50% oxidized.

FIG. 7 represents the oxidation of des-ala IL-2.sub.ser125 using o-phenanthroline/Cu.sup.+2 complex (FIG. 7B) compared with oxidation using CuCl.sub.2 (FIG. 7A).

FIG. 8 represents a graph of the percentage of des-ala IL-2.sub.ser125 oxidized (the disappearance of free sulfhydryl groups as measured by reaction with DTNB) as a function of time in minutes at 25.degree. C. using 50 .mu.M CuCl.sub.2, 0.25 mg/ml partially purified IL-2, 50 mM sodium phosphate,



0.1% SDS, at pH 7.0.

FIG. 9 represents a SDS-PAGE analysis of both a reducing and non-reducing gel of the HPLC-purified IL-2 oxidized using 50  $\mu$ M CuCl<sub>2</sub>.

FIG. 10 represents a RP-HPLC absorbance profile of the product of oxidation of des-alanyl IL-2 (containing the three cysteines present in native IL-2) promoted by 50  $\mu$ M CuCl<sub>2</sub>.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The recombinant proteins which are oxidized by the method of this invention are not native to the hosts used to produce them. Both IL-2 and  $\beta$ -IFN have amino acid sequences which are substantially identical to useful proteins and include cysteine residues which in the useful protein are linked intramolecularly to form a single cystine moiety (disulfide bridge). In this regard the term "substantially identical" means that the amino acid sequences of the recombinant and useful proteins are either identical or differ by one or more amino acid alterations (e.g., deletions, additions, or substitutions) which do not cause an adverse functional dissimilarity between the recombinant protein and its native counterpart. The recombinant proteins which are oxidized in the process of this invention are fully reduced, i.e., they lack disulfide bridging. For a protein such as interleukin-2 to be a uniform substrate for oxidation, it is usually reduced prior to the oxidation process. Reduction may be accomplished by treating the protein with a reducing agent such as dithiothreitol or 2-mercaptoethanol at an elevated temperature for a short period of time. The reducing agent is then removed immediately prior to the oxidation reaction.

The recombinant proteins to be oxidized by the process of this invention may be generated using established genetic engineering techniques. These techniques involve identifying and characterizing the structural gene which encodes the native protein, isolating or synthesizing that gene or a mutant which encodes a functionally equivalent mutein of the native protein, inserting the gene into an appropriate expression vector in a position which permits expression of the gene, transforming competent heterologous hosts, preferably microorganisms, with the vector, identifying correct transformants, and culturing the transformants in a suitable growth medium. The protein is typically recovered from the culture by disrupting the cells, treating the cellular debris with solubilizing agents (depending on the solubility characteristics of the protein) and one or more extractants to isolate crude protein, and purifying the crude protein by various preparative chromatography procedures. If the protein is susceptible to oligomer formation during the fermentation or recovery processes, the protein will be treated with a reducing agent at an appropriate stage in the recovery process.

After the recombinant protein is recovered from the host in a crude, substantially pure, or pure form, it is reduced and then oxidized with controlled kinetics using the invention process. Controlled oxidation pursuant to the process of this invention causes the formation of disulfide bridging in the recombinant protein which conforms to the bridging in its native counterpart with no or minimal overoxidation and no or minimal formation of incorrect disulfides or unwanted oligomers. Such oxidation enables the production of high yields of the recombinant protein in a configuration which most closely resembles the configuration of its native counterpart, thereby ensuring the likelihood that the recombinant protein will be functionally equivalent to the native protein.

The term "recombinant protein" as used herein also refers to muteins of IL-2 and  $\beta$ -IFN. Such muteins include, for example, proteins in which one or more cysteines not involved in disulfide bridging have been replaced with another amino acid to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation. Other IL-2 muteins in which amino acids besides cysteine have been replaced have also been constructed and are fully active.

A gene containing an undesirable and inessential cysteine can be selectively modified using a synthetic oligonucleotide primer complementary to the region of the gene but containing single or multiple base changes in the cysteine codon, resulting in a mutant protein (mutein) which now contains a different amino acid at that position. When deletion is desired the oligonucleotide primer would lack the codon for cysteine. Conversion of cysteine to neutral amino acids such as glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine is the preferred approach. Serine, threonine, or alanine are preferred replacements because of their chemical similarity to cysteine. When the cysteine is deleted, the mature mutein is one amino acid shorter than the native parent protein.

Human IL-2 and IFN- $\beta$ , both contain three cysteine residues in the mature protein. The presence of three cysteines means that upon reoxidation, these proteins may form one of three possible intramolecular disulfide bridges, only one of which corresponds to the correct bridge found in the native molecule. Muteins of IFN- $\beta$  and IL-2 in which inessential cysteines have been changed to serines are discussed in detail in Mark et al., (1984), PNAS (USA), 81, 5662-5666 and Wang et al., (1984), Science, 224, 1431-1433, respectively.

The oxidation promoter which is used in the process is an agent which is responsible for promoting the oxidation of cysteine residues preferentially and which contains a divalent copper cation. Other divalent cations such as Fe<sup>sup.+2</sup> are much less effective as oxidation promoters. The Cu<sup>sup.+2</sup> cation is found to produce pure oxidized protein with minimal by-products. The term "preferentially" indicates that the oxidation promoter (1) preferentially oxidizes the cysteines to form a disulfide bond with no or insignificant oxidation to higher levels, (2) preferentially oxidizes cysteines to form disulfide bridges which correspond to those present in the naturally occurring protein species, and (3) preferentially oxidizes cysteine residues as opposed to other residues. The oxidation promoter herein is capable of promoting oxidation of a mutein of IL-2 containing two cysteine residues so as to obtain at least 95% yield of the desired product with the cysteines oxidized to form disulfide bridges which correspond to those present in the naturally occurring protein. It is also capable of promoting oxidation of a mutein of IL-2 containing three cysteine residues so as to obtain at least 80-85% yield of the desired product. Examples of suitable oxidation promoters herein include CuCl<sub>2</sub> and (o-phenanthroline)<sub>2</sub> Cu<sup>sup.+2</sup> complex. Preferably, the oxidation promoter is CuCl<sub>2</sub>.

The amount of oxidation promoter employed is at least an effective amount for oxidation, i.e., an amount which at minimum will be necessary to conduct the oxidation reaction effectively within a convenient period of time. This amount, and the optimum amount for each reaction, may depend specifically on such factors as, for example, the type of protein, the type of oxidation promoter, the reaction temperature, the pH of the reaction, and the type and concentration of the solubilizing agent. Altering the concentration of oxidation promoter and time for oxidation is also expected to affect the types and amounts of side products generated. For pharmaceutical purposes it will usually be necessary to remove substantially all of the side products as well as unoxidized starting material which could theoretically generate unwanted oligomers through thiol-disulfide exchange reactions. In the examples below an effective amount is the amount approximately equivalent to the concentration of free sulfhydryl groups on the protein which are destined to be involved in forming the desired disulfide bonds. This amount, of course, must be optimized for each protein according to criteria which include, but are not limited to, convenient reaction time, types and amounts of side products, pH, etc. It is likely that the independent variables interact in such a way that there may be no unique optimum set of conditions for all proteins.

FIG. 5, which illustrates the effect of CuCl<sub>2</sub> concentration on the rate of IL-2 oxidation, shows that the observed oxidation rate increases as the concentration of CuCl<sub>2</sub> increases from 0.5 to 50  $\mu$ M.



The reaction rate has also been shown to increase two-fold when the reaction is carried out at 37.degree. C. rather than at room temperature (see Example 3). Thus, the reaction can be controlled to minimize potential for over-oxidation by simply adjusting the oxidation promoter concentration, the reaction time, or the reaction temperature. Preferably, the amount of CuCl.sub.2 will range from about 1 to 400 micromolar, depending on the protein concentration, more preferably 5 to 50 micromolar if the protein is IL-2.

The concentration of protein in the reaction mixture is generally kept relatively low to reduce the likelihood of oligomer formation. Depending on the sulfhydryl content and the molecular weight of the protein which is being oxidized, the protein concentration is generally less than about 5 mg/ml, preferably about 0.05 to about 2 mg/ml, and more preferably about 0.1 to about 1 mg/ml.

The pH of the reaction medium is generally maintained at a level of between about 5.5 and 9. Use of pHs significantly above the pH range specified herein causes a significant decrease in the rate of oxidation using CuCl.sub.2 as the oxidation promoter. The pH is preferably maintained between about 6 and 8, and more preferably about 7, as indicated by FIG. 6, which illustrates the effect of pH on the rate of IL-2 oxidation.

The reduced, cloned protein, which is less soluble than the oxidized form of the protein, generally must remain in solution, i.e., be in solubilized form, for effective oxidation to occur. Therefore, the reaction mixture will preferably also contain at least an effective amount of a solubilizing agent to prevent the protein from precipitating out of the solution. As used herein, the term "solubilizing agent" refers to an ionic or nonionic protein-solubilizing solute such as, e.g., sodium dodecyl sulfate (SDS) or urea. The amount of solubilizing agent which may be employed for this purpose is generally from about 0.1 to about 1% by weight per volume (for detergents) or about 5-9M (for urea), depending mainly on the protein and types of oxidation promoter used.

The oxidation reaction time will depend, for example, upon the concentration of reagents in the reaction mixture, the reaction temperature and the types of reagents. The reaction temperature will normally be between about 20.degree. C. and about 40.degree. C., conveniently room temperature, to maintain the solubilizing agent/protein mixture in solution. Increasing the reaction temperature increases the rate of reaction. For achievement of complete oxidation, the reaction time or temperature may be altered as appropriate for the particular process. The oxidation reaction may be effectively terminated by, for example, freezing the solution, adding chelators such as EDTA to the reaction mixture, or lowering the pH to a level at which the reaction ceases. Other factors such as concentration of solubilizing agent may also affect the rate of reaction. Following the reaction, residual oxidation promoter and undesired isomers or oligomers may be removed by selective ultrafiltration or chromatographic techniques. If necessary the oxidized protein may be purified further from side products and any residual reduced protein using protein purification procedures such as reverse phase high performance liquid chromatography (RP-HPLC). The extent of oxidation during the reaction is also readily quantifiable by RP-HPLC analysis.

Recombinant des-ala IL-2.sub.cys125 contains three cysteines and is theoretically susceptible to incorrect disulfide bond formation. When this protein is oxidized by the method described herein, the resulting product consists of protein having mostly the disulfide bridging of its native counterpart (between cysteines at residues 58 and 105 [Wang, et al. (1984) Science, 224:1431-1433; Robb, et al. (1984) PNAS, 81:6486-6490]). The oxidized protein is substantially free of oligomers (less than about 1-2% by weight) and contains less than about 15% by weight of biologically inactive isomers which have disulfide bridging different from that of the native counterpart. In contrast, preparations made via uncontrolled oxidations may contain significant amounts of oligomers (5%-10%) and even larger amounts of undesired isomers. Uncatalyzed air oxidations proceed slowly over a matter of days and are

very slow to reach completion.

Proteins which have been designed to eliminate the possibility of isomer formation (e.g., IL-2 in which the cysteine at position 125 has been changed to serine, or IFN-.beta. in which the cysteine at position 17 has been changed to serine), of course, contain no isomers. In the case of at least IL-2, the oxidized protein is much more water soluble than the reduced species and also has a higher specific activity in biological assays. Accordingly, the amount of the solubilizing agent (e.g., SDS) in the preparation may be decreased, generating a purified product which is sufficiently water soluble to permit formulation with conventional aqueous parenteral vehicles in a fashion suitable for use in humans or animals. In addition, this oxidized recombinant IL-2 mutein contains only the disulfide bridging present in IL-2 isolated from natural sources. The same procedure used above for the formation of disulfide bonds may be applied to other muteins of IL-2 to generate homogeneous, biologically active material.

Because the protein preparations prepared by the controlled oxidation typically contain more desired product and fewer by-products than preparations made via uncontrolled oxidation, they are expected to be less antigenic and possibly more therapeutically active.

Preparations of therapeutic proteins will comprise a therapeutically effective amount of the protein together with a pharmaceutically acceptable carrier. The preparation will generally be formulated for parenteral administration in vehicles such as distilled water, human serum albumin, and/or dextrose in water, or physiological saline.

The following examples further illustrate the invention process. These examples are not intended to limit the invention in any manner. In the examples all temperatures are in degrees Celsius.

#### EXAMPLE 1

##### Controlled Oxidation of Recombinant IFN-.beta..sub.ser17

##### Preparation of IFN-.beta..sub.ser17

IFN-.beta..sub.ser17 is a microbially produced mutein of IFN-.beta. in which the cysteine residue at amino acid position 17 is replaced with a serine residue. IFN-.beta..sub.ser17 has two remaining cysteine residues: one at position 31 and the other at position 141. In native IFN-.beta. the cysteines at positions 31 and 141 interact to form a disulfide bridge. The genetically engineered E. coli strain used in this example to produce IFN-.beta..sub.ser17 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 USA on Nov. 18, 1983 under accession No. 39,517.

The genetically engineered E. coli mentioned above was grown in the following medium:

	Approximate Initial Ingredient Concentration
Na.sub.3 Citrate.2H.sub.2 O	3 mM
H.sub.2 PO.sub.4	30 mM
(NH.sub.4).sub.2 SO.sub.4	74 mM
MgSO.sub.4.7H.sub.2 O	3 mM
MnSO.sub.4.H.sub.2 O	46 .mu.M
ZnSO.sub.4.7H.sub.2 O	46 .mu.M
CuSO.sub.4.5H.sub.2 O	1-2 .mu.M
L-tryptophan	350 .mu.M
FeSO.sub.4.7H.sub.2 O	74 .mu.M
thiamine.HCl	0.002% (w/v)
glucose	0.5% (w/v)

A 25% solution of Dow Corning Antifoam B, a 50% solution of glucose and 5N KOH were added on demand.

The temperature was maintained at 37.+-.1.degree. C., the pH at 6.5.+-.0.1 with NaOH, and dissolved

oxygen at 30% w/w of air saturation. Optical density and residual glucose measurements were taken at 14 hours and at approximately one-hour intervals thereafter. Harvest was consumed reached 40. $\pm$ .6 g/l (OD at 680 nm=10-11).

The harvested material was concentrated approximately 3-fold by circulating it through a microporous cross-flow filter under pressure. The concentrated cells were diafiltered against deionized water until the harvest material was concentrated 4-5 fold. The cells were then disrupted by passing them through a Manton-Gaulin homogenizer at 4.1-5.5.times.10.sup.4 kpa (0.6-0.8 psi). After the initial pass sodium dodecyl sulfate (SDS)-sodium phosphate buffer was added to a final concentration of 2% w/v SDS, 0.08M sodium phosphate, and solubilization was continued for one hour. Solid dithiothreitol (DTT) was then added to a final concentration of 50 mM and the homogenate was treated to 90. $\pm$ .5.degree. C. for 10 minutes. The resulting cell suspension was extracted with 2-butanol at a 1:1 2-butanol:suspension volume ratio in a static mixer. The mixture was then centrifuged and the 2-butanol-rich phase was collected.

The 2-butanol-rich phase was mixed with 2.5 volumes of 0.1% w/v SDS in phosphate buffered saline (PBS). Solid DTT was added to a final concentration of 1 mM. The pH of the mixture was adjusted to 6.2. $\pm$ .0.1 with glacial acetic acid and this mixture was centrifuged. The resulting paste was collected and resuspended in a mixture of PBS and 10% w/v SDS with pH adjustment to 8.5. $\pm$ .0.1 using 1N NaOH. Solid DTT was added to a final concentration of 100 mM and the suspension was heated to 90. $\pm$ .5.degree. C. for 10 minutes. The suspension was then cooled to about 25.degree. C., the pH was adjusted to 5.5. $\pm$ .0.1 with glacial acetic acid, and the solution was filtered.

The solution was then applied to a Sephacryl S-200 precolumn with a buffer consisting of 1% SDS, 50 mM sodium acetate, 1 mM EDTA, pH 5.5. The fractions containing highest interferon activities were pooled and concentrated by ultrafiltration with a 10 kilodalton molecular weight cut-off.

The protein was oxidized to generate sulfhydryl bonds using the method of Shaked, et al., supra. A 1 mM o-iodosobenzoic acid solution was prepared by mixing the acid in water, sonicating the mixture for about 5 minutes and then stirring and adding 2% NaOH slowly to obtain a final pH of 8.2. $\pm$ .0.2 (additional sonication may be used as an alternative to adding base).

A reaction buffer medium was prepared by dissolving Na.sub.4 P.sub.2 O.sub.7.10H.sub.2 O in water to a concentration of 2 mM. The pH of this solution was adjusted to 9.0 by adding 10% acetic acid, SDS to 0.1%, ethylenediaminetetraacetic acid (EDTA) to 1 mM and the o-iodosobenzoic acid solution to 15 .mu.M were added to the solution.

The buffer medium was placed in a reaction vessel equipped with a magnetic stirrer and a pH electrode set at 9.0. The IFN-.sub.ser17 preparation and the o-iodosobenzoic acid solutions were added to the reaction mixture from holding vessels using peristaltic pumps that were calibrated to introduce equivalent mole ratios of the IFN and oxidizing agent. The pH of the reaction mixture was controlled at 9.0 by adding 0.25M NaOH via a peristaltic pump at 5 ml/hr as needed. The IFN-.beta. solution (5 mg/ml in 50 mM acetate buffer, pH 5.5) was added at a flow rate of 2 ml/hr (7.0 micromole/hr) for about 5 hours; the o-iodosobenzoic acid solution was added at 7 ml/hr (7 micromole/hr) over the same time period. The addition of the acid solution was continued thereafter to get a final excess of 10-15 micromolar. The reaction was followed by reverse phase HPLC and by assaying the residual thiol content of IFN-.beta..sub.ser17 by Ellman's assay. After 6.5 hours the reaction was terminated by adding 10% acetic acid to the reaction mixture to a pH of 5.5.

The product was then loaded on a Sephacryl-200 column using a buffer consisting of 0.1% SDS, 1 mM EDTA, and 50 mM sodium acetate at pH 5.5. The monomer peak from this column was pooled and

loaded on a Sephadex G-75 column using a buffer consisting of 0.1% SDS, 1 mM EDTA, and 50 mM sodium acetate at pH 5.5.

#### Oxidation of Fully Reduced IFN-.beta..sub.ser17

The Sephadex G-75 pooled material which had been oxidized using the above iodosobenzoic acid oxidation method was employed for the following copper oxidation studies because it was the only interferon product readily available. The purified IFN-.beta..sub.ser17 was reduced for 15 minutes at 50.degree. C. following addition of dithiothreitol to 10 mM to ensure that none of the molecules contained disulfide linkages. This was confirmed by RP-HPLC in a 30-60% acetonitrile gradient (30-40% in 5 min, 40-60% in 27 min) in 0.1% v/v trifluoroacetic acid (using a Vydac C4 column), which separates the oxidized from the reduced form of interferon (retention times 26 and 28 min, respectively). The reduced .beta.-interferon was concentrated from the RP-HPLC peak fraction by lyophilization and resuspension in 0.1% w/v SDS and 50 mM phosphate buffer at pH 7.0 containing 5 micromolar EDTA. The reaction mixture contained 0.13 mg/ml of the interferon. Oxidation was initiated by adding CuCl.sub.2 to a final concentration of 8 micromolar using air-saturated solutions at 25.degree. C.

It has been shown by others that the kinetics of disulfide formation can be measured by monitoring changes in elution position from various HPLC columns (Wu, et al., Anal. Biochem., 129, 345-348 (1983) and Reference 8 therein). One assay for oxidation used in the present examples relies on a shift in elution position on RP-HPLC following oxidation.

FIG. 1A illustrates RP-HPLC of a control reaction containing the reduced .beta.-interferon after 7 minutes in the resuspension media without CuCl.sub.2. FIG. 1B illustrates RP-HPLC of the reaction mixture containing CuCl.sub.2 after 7 minutes of oxidation, FIG. 1C, after 14 minutes of oxidation, FIG. 1D, after 28 minutes of oxidation, and FIG. 1E, after 75 minutes. FIG. 1F illustrates a plot of percent oxidation versus minutes of oxidation based on the RP-HPLC analysis. The results show that the interferon is more than 95% oxidized by 75 minutes. This oxidized product was assayed for anti-viral activity using the cytopathic effect assay described by Steward, W. E. II, The Interferon System, (New York:Springer-Verlag, 1981), p. 17, and was found to have the same specific bioactivity as native .beta.-IFN, 1.times.10.sup.8 units/mg.

The sample which was oxidized for 75 minutes was then reduced in 10 mM DTT at 50.degree. C. for 15 minutes. FIG. 2A shows the RP-HPLC of the oxidized material and FIG. 2B shows the RP-HPLC of the reduced material. Comparison of the chromatograms indicates that the shift in RP-HPLC retention time was due to an oxidation which was reversible by DTT reduction.

#### EXAMPLE 2

##### Controlled Oxidation of Des-Ala IL-2.sub.ser125

##### Preparation of Fully Reduced Des-Ala IL-2.sub.ser125

Des-ala IL-2.sub.ser125 is an IL-2 whose amino acid sequence differs from native human IL-2 by: (1) the absence of the initial N-terminal alanine residue and (2) a serine substituted for cysteine at position 125. The strain of des-ala IL-2.sub.ser125 -producing E. coli used for this example was deposited in the ATCC on Mar. 6, 1984 under accession No. 39,626.

The genetically engineered E. coli mentioned above was grown in a fermenter using the following growth medium:



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Approximate Initial Concentration  
 Initial Ingredients Added: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 72 mM KH<sub>2</sub>PO<sub>4</sub> 21.6 mM Na<sub>3</sub>Citrate 1.5 mM ZnSO<sub>4</sub>·7H<sub>2</sub>O 60 . $\mu$ M MnSO<sub>4</sub>·H<sub>2</sub>O 60 . $\mu$ M CuSO<sub>4</sub>·5H<sub>2</sub>O 2 . $\mu$ M pH adjusted to 6.50 with 2.5 N NaOH Autoclaving Sterile Additions (post autoclave): MgSO<sub>4</sub>·7H<sub>2</sub>O 3 mM FeSO<sub>4</sub> 100 . $\mu$ M L-tryptophan 70 mg/l Thiamine-HCl 20 mg/l Glucose 5 g/l Tetracycline 5 mg/l Ethanol (optional) 2% (w/v) Casamino acid 2% (w/v)

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A 20% solution of Dow Corning Antifoam B, a 50% solution of glucose and 5N KOH were added on demand.

The pH of the fermenter was maintained at 6.8 with 5N KOH. Residual glucose was maintained between 5-10 g/l, dissolved oxygen at 40% w/w, and temperature at 37. $\pm$ 1.degree. C. The casamino acids (20% w/v stock solution) were added when the OD<sub>680</sub> was about 10-15. Three hours after adding the casamino concentrated solution, ethanol (95% w/w) was added to achieve a final 2% w/w concentration. Harvest was made two hours after ethanol addition.

About 20-40 g (wet weight) of the E. coli MM294-1 cells containing the induced, cloned IL-2 were resuspended in 200 ml of 50 mM Tris, 1 mM EDTA (pH 8.1 to 8.5). The high pH aided in selective extraction of E. coli proteins in subsequent steps. The cells were centrifuged at 3000-4000.times.g for 10 minutes and resuspended in 200 ml of Tris/EDTA buffer at 4.degree. C.

The cells were then sonicated at 4.degree. C. for 45 minutes (or until the optical density at 600 had dropped about 85%) using large probe pulsing with 50% duty on power setting "9" of Heat Systems Model W-375 sonicator. Alternatively the cells were broken by three passes through a Manton-Goulin homogenizer. The homogenate was centrifuged at 4500.times.g for 10 minutes (6000 rpm) using a Beckman JA20 rotor at 4.degree. C. The debris was resuspended in 60 ml of a Tris/EDTA mixture at room temperature. Over a period of 5 minutes an equal volume of 8M urea (Schwartz/Mann ultra pure) in Tris/EDTA buffer was added to the suspension with rapid stirring to yield a final urea concentration of 4M. The resulting mixture was stirred slowly for 15-30 minutes at room temperature.

After stirring the mixture was centrifuged at 12,000.times.g for 15 minutes (12,000 rpm in Beckman JA20 rotor at room temperature) and the pellet was saved. The pellet was then resuspended in 9 ml of 50 mM sodium phosphate (at pH 6.8), 1 mM EDTA, 10 mM DTT at 20.degree. C. The pellet was then solubilized by addition of 1 ml of 20% w/v SDS and vortexed vigorously. The resuspension was centrifuged at 12,000.times.g for 10 minutes at room temperature and the insoluble material was discarded.

The remaining solution was heated to 40.degree. C. for 15 minutes to ensure that all of the IL-2 was fully reduced. The supernatant fluid (containing 40% pure IL-2) was loaded onto a 2.6 cm.times.100 cm Sephacryl-200 (S-200) column run in 50 mM sodium phosphate (pH 6.8), 1 mM EDTA, 1 mM DTT, 1% w/v SDS. Then 3 . $\mu$ l aliquots of each fraction were run on a 15% w/v SDS/PAGE minigel and the gel was stained with Coomassie blue. The fractions with the fewest contaminants (minimizing the inclusion of contaminants at about 35K, 18K and 12K daltons) were pooled and concentrated to 5-10 ml using an Amicon YM5 ultrafilter. The preparation was about 80-90% pure IL-2.

The S-200 pool was loaded onto a 2.6 cm.times.100 cm Sephadex G-100 column, which was eluted as described above using 0.1% w/v SDS. The fractions were analyzed by SDS/PAGE and the purest fractions pooled. These purest fractions contained 95-98% pure IL-2 with 0.2-0.5 ng of endotoxin per 100,000 units. Over 30% of the IL-2 present in the crude lysate was recovered as pure IL-2.

When stored at 4.degree. C. under nitrogen these pooled G-100 fractions were found to be stable for at least six weeks without additions. An SDS-containing precipitate formed at 4.degree. C. which could be redissolved at 25.degree. C. prior to use or could be removed without significant loss of IL-2 units.

#### Controlled Oxidation of Des-Ala IL-2.sub.ser125 Using CuCl.sub.2

The purified, fully reduced product obtained as described above was adjusted to 0.5 mg/ml IL-2 by diafiltration/ultrafiltration against 50 mM Tris-HCl buffer at pH 8.0. Diafiltration against Tris buffer acted not only to adjust pH but also removed any remaining EDTA or DTT which might interfere with the oxidation reaction. The SDS concentration after the diafiltration/ultrafiltration step was 1.6% w/v (as measured by the acridine orange assay for SDS described in Anal. Biochem., Vol. 118, p. 138-141 (1981)), and the pH was about 8.0. The concentrate was oxygenated by bubbling air through the solution, and the oxidation was initiated by adding CuCl.sub.2 to 0.5 .mu.M, 5 .mu.M, or 50 .mu.M using freshly prepared solutions. Reactions were carried out at 25.degree. C. For determining the kinetics of oxidation, aliquots of the reaction mixtures were taken at various time intervals and quenched by adding EDTA up to 10 mM concentration and quick freezing at -70.degree. C. Each aliquot was analyzed by RP-HPLC using acetonitrile gradient elution (30-60% in 45 min) in 0.1% trifluoroacetic acid to determine the extent of reaction, because RP-HPLC separates the oxidized from the reduced form of the IL-2 (retention times 41 and 45 min, respectively).

FIG. 3 shows the RP-HPLC analysis of the oxidation reactions using 50 .mu.M CuCl.sub.2 (before and after re-reduction). FIG. 3A shows that after only 2 minutes of oxidation the sample was approximately two-thirds oxidized. FIG. 3B shows that after 10 minutes of oxidation the sample was essentially entirely oxidized, and only traces of other peaks (indicative of side reactions) were observed. FIG. 3C shows that 50 minutes of oxidation did not increase the amounts of the minor product components produced, and therefore that side reactions do not occur during prolonged oxidation. FIG. 3D shows that the oxidation product after 50 minutes of oxidation can be re-reduced to the fully reduced IL-2 using 10 mM dithiothreitol for 15 minutes at 60.degree. C. This indicates that the peaks in FIGS. 3A, 3B and 3C represented oxidized material which was reducible.

FIG. 4 shows a silver-stained non-reducing SDS-PAGE analysis of the product of 40 minutes of oxidation using 50 .mu.M CuCl.sub.2. Despite the sensitive staining technique employed, only a trace of the oxidized product was found to have formed intramolecular sulfhydryl groups, generating dimers.

The studies of oxidations performed at various CuCl.sub.2 concentrations are summarized graphically in FIG. 5. This figure shows that the observed oxidation rate as measured by HPLC peak height, less background, is dependent on CuCl.sub.2 concentration, and therefore the reaction can be controlled by adjusting this parameter. The reaction using 5 .mu.M CuCl.sub.2 contained a 6-fold molar excess of IL-2, yet was completely oxidized in 60 minutes, indicating that the CuCl.sub.2 may act catalytically. A control reaction, containing 10 mM EDTA, showed essentially no oxidation.

A similar series of oxidations was conducted on a 0.2 mg/ml IL-2 solution using 8 .mu.M CuCl.sub.2 in a mixture of 30 mM Tris and 30 mM sodium phosphate buffer adjusted to pH 6, 6.5, 7.5, 8.0, 8.5 or 9.5 and containing 0.1% w/v SDS. In all reactions the pH was confirmed at the end of the reaction. The studies of oxidations performed at these pH values for 7 minutes are summarized graphically in FIG. 6. This figure shows that there is an optimum pH range for oxidation of IL-2: about 6 to 8, above which the oxidation rate falls off dramatically.

#### Controlled Oxidation of Des-Ala IL-2.sub.ser125 Using (o-Phenanthroline).sub.2 Cu.sup.+2 Complex

The above oxidation procedure was repeated using a freshly prepared 8 .mu.M (o-phenanthroline).sub.2



Cu.sup.+2 complex instead of CuCl.sub.2. The Tris/phosphate buffer described above was employed at pH 7.0, containing 0.1% SDS (w/v). FIG. 7 gives a comparison of the RP-HPLC for the IL-2 after 7 minutes of oxidation with 8 .mu.M CuCl.sub.2 (FIG. 7A) with the RP-HPLC for the IL-2 after 7 minutes of oxidation with 8 .mu.M (o-phenanthroline).sub.2 Cu.sup.+2 (FIG. 7B). The results show that IL-2 can be more rapidly oxidized by (o-phenanthroline).sub.2 Cu.sup.+2 complex at pH 7 than by CuCl.sub.2 alone.

#### Purification of Oxidized Des-Ala IL-2.sub.ser125

The insoluble material recovered from the urea extraction of Example 2 was resuspended in 50 mM sodium phosphate buffer, 1 mM EDTA pH 7.0. The suspension was then solubilized by addition of solid SDS to a final concentration of 5% w/v.

The 5% SDS solution was diluted to 2% SDS with 0.1M Na.sub.2 PO.sub.4, pH 8.0. The protein concentration was determined, the pH was adjusted to 8.5, and DTT to 50 mM and EDTA to 2 mM were added. The mixture was heated to 40.degree. C. under N.sub.2 to reduce the IL-2. The mixture was then cooled and the pH was adjusted to 5.0.

The solution was then extracted at a 1:1 ratio (v/v) with 2-butanol containing 1 mM DTT at room temperature. Residence time was 2-2.5 minutes. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic extract was separated and its pH was adjusted to 8.0 with NaOH. The extract was then added slowly to 0.1% SDS in 10 mM Na.sub.2 PO.sub.4, 2 mM DTT, pH 6 and stirred for 15-20 minutes. The resulting precipitate was separated and the resulting paste was resuspended in 5% SDS in PBS. The solution was clarified by centrifugation and reduced as above. Following reduction the solution was adjusted to pH 5.5 with acetic acid. The solution was purified by gel filtration using a 2.6 cm.times.100 cm S-200 column run in 50 mM sodium phosphate (pH 6.8), 1 mM EDTA, 1 mM DTT, 1% w/v SDS.

The peak fractions from this column were pooled, and a portion of this material (in 50 mM sodium acetate (pH 5.5), 1% SDS, 2 mM DTT and 1 mM EDTA) was concentrated to 760 microliters using an Amicon YM-5 ultrafiltration membrane, yielding 6.6 mg of total protein. Dithiothreitol was added to a concentration of 2.5 mM, and the sample was heated to 60.degree. C. for 10 minutes to ensure full reduction. Reducing agent was removed using a G-25 desalting column (19.times.0.9 cm) equilibrated in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1% SDS. The protein peak was pooled, yielding 5.5 mg, which was diluted to 0.25 mg/ml in column buffer. The amount of free sulfhydryl groups was immediately assayed using the Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid), (DTNB)) sulfhydryl assay (as described by Habeeb, A.F.S.A., Methods of Enzymology, vol. 25, Part B, pp 457-64 (1972) using cysteine as a standard).

Air was bubbled through the sample for 15 seconds to aerate the solution, and oxidation at 25.degree. C. was initiated by addition of CuCl.sub.2 to a concentration of 50 micromolar. The extent of oxidation was measured by assaying residual free sulfhydryl groups essentially using the DTNB assay after 5, 10, and 30 minutes of room temperature incubation.

FIG. 8, which depicts the kinetics of oxidation of the peak IL-2 fractions from the S-200 column, shows that oxidation was essentially complete by 30 minutes. EDTA was added to a concentration of 10 mM at 35 minutes, followed by addition of one-tenth volume of 100% acetonitrile/5% trifluoroacetic acid. The oxidized IL-2 was then separated from remaining E. coli protein and endotoxin by preparative RP-HPLC on a 10 mm.times.25 cm, 10 micron Vydac C4 column equilibrated in 10% acetonitrile/0.1% TFA. IL-2 was recovered at 60% acetonitrile following gradient elution at 2 ml/min. (10-30% acetonitrile in 5 minutes, 30-60% acetonitrile in 45 minutes). The peak of IL-2 was pooled, and the total

protein recovered was determined to be 3 mg by absorption at 280 nm.

At this point the protein was formulated as follows: to a volume of 7.7 ml pooled HPLC fractions mannitol was added to 1.7%, and SDS was added to 0.037%. The sample was lyophilized overnight and resuspended in 2.9 ml of 50 mM sodium phosphate (pH 6.8) in WFI (water for injection). The final concentrations of SDS and mannitol were 0.1% and 5%, respectively.

Four micrograms of the lyophilized, resuspended IL-2 was subjected to SDS-PAGE minigel analysis under reducing and non-reducing conditions (boiling 5 minutes in 2% SDS, 50 mM tris-HCl, pH 6.8, with or without 1%  $\beta$ -mercaptoethanol). The analysis, given in FIG. 9, shows that the non-reduced IL-2 migrated slightly faster than the reduced IL-2, as expected from the literature for a molecule containing disulfide bonds. Densitometric scans using the Shimadzu CS-930 scanner of the TCA-fixed, Coomassie-stained gel showed that the final product is over 95% pure and contains less than 2% protein migrating in the positions expected for oligomeric IL-2. Where necessary, residual IL-2 oligomers can be effectively eliminated by molecular sieve chromatography (S-200 columns, run as described above) following the IL-2 oxidation step. The RP-HPLC appeared to remove both residual *E. coli* contaminants and pyrogens. At least 50% of the pooled S-200 starting material was recovered in the final oxidized product.

The final specific bioactivity of the purified IL-2 was measured to be 4-6.times.10.sup.6 units/mg (units based on units measured with the HT-2 cell proliferation assay (Watson, J. (1979) JEM, 150:1510-1519 and Gillis, S., et al. (1979) J.I., 120:2027-2032) and on protein content measured by the method of Lowry, O. H., et al. (1951) J. Biol. Chem., 193:265-275), and the endotoxin content was less than 0.3 nanograms/mg IL-2, as measured by limulus amoebocyte lysate (LAL) assay. The specific biological activity of the purified oxidized product is essentially indistinguishable from that of native IL-2 from induced peripheral blood lymphocytes or the native IL-2 isolated from the induced Jurkat cell line. Thus, the oxidized, recombinant IL-2 bioactivity resembles that of two native counterparts which are known to be oxidized. Reduced native and reduced recombinant IL-2 proteins both have significantly lower specific bioactivities. Because the oxidized product herein was shown to have biological activities identical to those measured for native Jurkat or peripheral blood lymphocyte IL-2 (unpublished observations), IL-2 produced by the present process may be useful in enhancing the ability of the human immune system to combat viral pathogens and cancers.

The final product may be lyophilized for storage, and/or may be stored in solution at 4.degree. C. for up to 60 days or more without significant change in biological or physical properties.

When the oxidation reaction of des-ala IL-2.sub.ser125 was conducted as described above but without using SDS or another solubilizing agent to keep the fully reduced IL-2 in solution, no measurable oxidation took place. When the oxidation was carried out on des-ala IL-2.sub.ser125 using the preferred conditions described above except using FeSO.sub.4 as the oxidation promoter, less than 10% of the product had been oxidized, indicating that Fe.sup.+2 is much less effective a cation for promoting oxidation.

When the oxidation was carried out on a reduced molecule containing three cysteines (i.e., des alanyl, recombinant IL-2 produced from an *E. coli* strain deposited in the American Type Culture Collection on Aug. 4, 1983 under accession no. 39,405) using the conditions described above, at least 85% of the product had the correct disulfide linkage (between cys 58 and cys 105) and showed identical bioactivity to the native protein. Approximately 15% of the material was inactive, presumably representing isomers of IL-2 which contain incorrect disulfide linkages. FIG. 10 shows a RP-HPLC analysis of the final product eluted with a 30-60% acetonitrile gradient. The retention times are 29 min for inactive isomers and 44 min for active IL-2.

### EXAMPLE 3

Controlled Oxidation of Des-Ala IL-2.sub.ser125 at 37.degree. C.

This example illustrates the effect of increased temperature on the oxidation reaction of this invention.

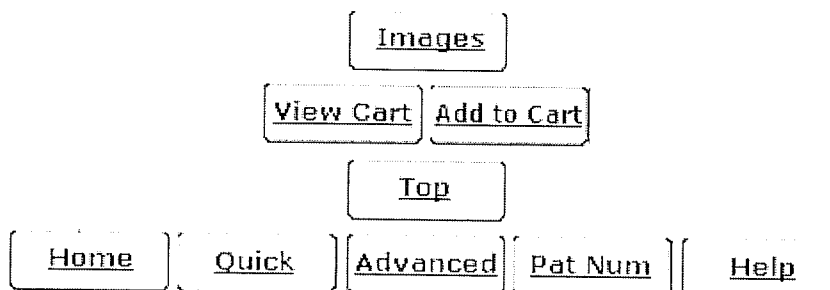
The peak fractions from the S-200 column described in Example 2 under Purification of Oxidized Des-Ala IL-2.sub.ser125 were pooled and the peak material was concentrated to 720 microliters using an Amicon YM-5 ultrafiltration membrane. Dithiothreitol was added to a concentration of 3.5 mM, and the IL-2 was then heated to 60.degree. C. for 10 minutes. The dithiothreitol was then removed using a G-25 desalting column equilibrated in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1% SDS. The protein concentration was adjusted to 0.25 mg/ml using the same buffer. The IL-2 was then divided into two 10-ml portions and equilibrated at either 25.degree. C. or 37.degree. C. in a circulating water bath. The oxidation was initiated by adding CuCl.sub.2 to 50 .mu.M. The kinetics of oxidation were then determined by (1) measuring the amount of free sulfhydryl groups using the Ellman's reagent assay, and (2) monitoring the amount of IL-2 present at the retention times expected for reduced and oxidized IL-2 following RP-HPLC.

Raising the temperature of oxidation from 25.degree. C. to 37.degree. C. increased the rate of oxidation approximately two-fold as measured by both assays. Thus, temperature, as well as copper concentration, has an effect on oxidation rate. Both the oxidations at 25.degree. C. and 37.degree. C. proceeded to essentially 100% completion, thereby minimizing free sulfhydryl groups and greatly diminishing the possibility of subsequent oligomer formation by thio-disulfide exchange. Reverse phase HPLC analysis revealed that the oxidized IL-2 products appeared identical, and bioassay showed that they had the same specific activity. Non-reducing SDS-PAGE analysis demonstrated that less than 1% oligomers had been formed at either temperature.

In summary, the present invention is seen to provide a controlled method of oxidizing fully reduced cysteine-containing recombinant IL-2 and IFN-.beta. using an oxidation promoter containing Cu.sup.+2 which catalyzes the in vitro formation of disulfide bridges which correspond to those found in the native proteins. The process herein eliminates or minimizes side reactions during the oxidation and maximizes the ability of the oxidized product to regain full bioactivity.

Modification of the above-described modes for carrying out the invention that are obvious to those of skill in biochemical engineering are intended to be within the scope of the following claims.

\* \* \* \* \*



**EXHIBIT P**  
**PATENT 4,853,332**

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( 1 of 1 )

**United States Patent****4,853,332****Mark , et al.****August 1, 1989**

Structural genes, plasmids and transformed cells for producing cysteine depleted muteins of biologically active proteins

**Abstract**

Muteins of biologically active proteins such as IFN-.beta. and IL-2 in which cysteine residues that are not essential to biological activity have been deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bridge formation. These muteins are made via bacterial expression of mutant genes that encode the muteins that have been synthesized from the genes for the parent proteins by oligonucleotide-directed mutagenesis.

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**Related U.S. Patent Documents**

<u>Application Number</u>	<u>Filing Date</u>	<u>Patent Number</u>	<u>Issue Date</u>
564224	Dec., 1983	4518584	
486162	Apr., 1983		
435154	Oct., 1982		

**Foreign Application Priority Data**

Oct 10, 1983 [IE]	2380/83
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**435/252.33 ; 435/252.1; 435/320.1; 435/69.52; 536/23.5;  
536/23.51; 930/141; 930/142**

**Current International Class:** C12N 15/10 (20060101); C07K 14/435 (20060101); C07K 14/565 (20060101); C07K 14/55 (20060101); A61K 38/00 (20060101); C12N 001/20 (); C12N 015/00 (); C07H 021/04 ()

**Field of Search:** 435/68,172.3,253,317,240.2,240.4,255,252.33,252.1 536/27

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### Parent Case Text

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### CROSS-REFERENCE TO RELATED APPLICATION

This application is division of U.S. Ser. No. 564,224 filed Dec. 20, 1983, now U.S. Pat. No. 4,518,584, which in turn is a continuation-in-part of U.S. Ser. No. 486,162 filed Apr. 15, 1983, now abandoned, which is a continuation-in-part of U.S. Ser. No. 435,154 filed Oct. 19, 1982, now abandoned.

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### Claims

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We claim:

1. A structural gene having a DNA sequence that encodes a synthetic interleukin-2 mutein wherein the cysteine residue at position 125, numbered in accordance with native human interleukin-2, is replaced by a neutral amino acid.
2. The structural gene of claim 1 wherein the neutral amino acid is selected from the group consisting of serine, threonine, glycine, alanine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan, and methionine.
3. The structural gene of claim 1, wherein the neutral amino acid is serine or threonine.
4. The structural gene of claim 1, wherein the neutral amino acid is serine.
5. The structural gene of claims 1, 2, 3, 4 with or without an initial ATG codon.
6. The structural gene as represented in FIG. 15a, with or without the initial ATG codon.
7. An expression vector that includes the structural gene of claims 1, 2, 3, 4 or 6.
8. The expression vector of claim 7, wherein the vector is pBR322 or pCR1.
9. The plasmid pLW46 having an ATCC accession number 39,452.
10. The plasmid pLW55 having an ATCC accession number 39,516.
11. A host cell transformed with an expression vector that includes a structural gene having a DNA sequence that encodes a synthetic interleukin-2 mutein wherein the cysteine residue at position 125, numbered in accordance with native human interleukin-2, is replaced by a neutral amino acid and said mutein exhibits the biological activity of native human interleukin-2, wherein the host cell is selected from the group consisting of bacteria, yeast, animal, and plant.
12. The host cell of claim 11, wherein the host cell is bacteria.
13. The host cell of claim 12, wherein the bacteria is *E. coli*.
14. The host cell of claims 11, 12 or 13, wherein the neutral amino acid is selected from the group consisting of serine, threonine, glycine, alanine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan, and methionine.
15. The host cell of claims 11, 12 or 13, wherein the neutral amino acid is serine.
16. *E. coli* transformed by an expression vector which includes the structural gene represented by FIG. 15a, with or without the initial ATG codon.
17. *E. coli* transformed with a plasmid selected from the group consisting of pLW46 and pLW55, and progeny thereof.

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*Description*

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## DESCRIPTION

### 1. Technical Field

This invention is in the general area of recombinant DNA technology. More specifically it relates to mutationally altered biologically active proteins that differ from their parent analogs by one or more substitutions/deletions of cysteine residues.

### 2. Background Art

Biologically active proteins that are microbially produced via recombinant DNA (rDNA) technology may contain cysteine residues that are nonessential to their activity but are free to form undesirable intermolecular or intramolecular links. One such protein is microbially produced human beta interferon (IFN-.beta.). In the course of the preparation of IFN-.beta. by rDNA techniques, it has been observed that dimers and oligomers of microbially produced IFN-.beta. are formed in E. coli extracts containing high concentrations of IFN-.beta.. This multimer formation renders purification and separation of IFN-.beta. very laborious and time-consuming and necessitates several additional steps in purification and isolation procedures such as reducing the protein during purification and reoxidizing it to restore it to its original conformation, thereby increasing the possibility of incorrect disulfide bond formation. Furthermore, microbially produced IFN-.beta. has also been found to exhibit consistently low specific activity due perhaps to the formation of multimers or of random intramolecular disulfide bridges. It would be desirable, therefore, to be able to alter microbially produced biologically active proteins such as IFN-.beta. in a manner that does not affect their activity adversely but reduces or eliminates their ability to form intermolecular crosslinks or intramolecular bonds that cause the protein to adopt an undesirable tertiary structure (eg, a conformation that reduces the activity of the protein).

The present invention is directed to producing by directed mutagenesis techniques mutationally altered biologically active proteins (such proteins are called "muteins", Glossary of Genetics and Cytogenetics, 4th ED, p 381, Springer-Verlag (1976)) that retain the activity of their parent analogs but lack the ability to form intermolecular links or undesirable intramolecular disulfide bonds. In this regard Shepard, H. M., et al, Nature (1981) 294: 563-565 describe a mutein of IFN-.beta. in which the cysteine at position 141 of its amino acid sequence (there are three cysteines in native human IFN-.beta. at positions 17, 31, and 141, Gene (1980) 10: 11-15 and Nature (1980) 285: 542-547) is replaced by tyrosine. This mutein was made by bacterial expression of a hybrid gene constructed from a partial IFN-.beta. cDNA clone having a G.fwdarw.A transition at nucleotide 485 of the IFN-.beta. gene. The mutein lacked the biological activity of native IFN-.beta. leading the authors to conclude that the replaced cysteine was essential to activity.

Directed mutagenesis techniques are well known and have been reviewed by Lather, R. F. and Lecoq, J. P. in Genetic Engineering Academic Press (1983) pp 31-50. Oligonucleotide-directed mutagenesis is specifically reviewed by Smith, M. and Gillam, S. in Genetic Engineering: Principles and Methods, PLenum Press (1981) 3: 1-32.

## DISCLOSURE OF THE INVENTION

One aspect of the invention is a synthetic mutein of a biologically active protein which protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutein having at least one of said cysteine residues deleted or replaced by another amino acid.

Another aspect of the invention relates to synthetic structural genes having DNA sequences that have

been specifically designed ("designer genes") to encode the above described synthetic muteins. Subaspects of this aspect are expression vectors that include such structural designer genes, host cells or organisms transformed with such vectors, and processes for making the synthetic mutein by culturing such transformants or their progeny and recovering the mutein from the culture. In the case of muteins that have therapeutic utility, therapeutic compositions that contain therapeutically effective amounts of the muteins and therapeutic methods are other aspects of the invention.

Another aspect of the invention is a method of preventing a protein having one or more cysteine residues that is free to form an undesirable disulfide link from forming such a link comprising mutationally altering the protein by deleting the cysteine residue(s) or replacing them other amino acids.

Still another aspect of the invention is a method for making the above described synthetic structural gene by oligonucleotide-directed mutagenesis comprising the following steps:

- (a) hybridizing single-stranded DNA comprising a strand of a structural gene that encodes the parent protein with a mutant oligonucleotide primer that is complementary to a region of the strand that includes the codon for the cysteine to be deleted or replaced or the antisense triplet paired with the codon, as the case may be, except for a mismatch with that codon or antisense triplet, as the case may be, that defines a deletion of the codon or a triplet that encodes said other amino acid;
- (b) extending the primer with DNA polymerase to form a mutational heteroduplex; and
- (c) replicating the mutational heteroduplex.

The mutant oligonucleotide primers used in this process are another aspect of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram of the amino acid sequence of IFN-.beta..

FIG. 2 is a schematic illustration showing the preparation of mutant IFN-.beta. gene by oligonucleotide-directed mutagenesis.

FIG. 3 shows a diagram of plasmid p.beta.ltrp including the IFN-.beta. gene.

FIG. 4 is a diagram of the cloning vector M13mp8 phage.

FIG. 5 shows the restriction map of clone M13-.beta.1.

FIG. 6 shows the sequencing gel pattern of the mutant IFN-.beta..sub.ser17 gene showing a single base change in the coding region.

FIG. 7 is a diagram of the expression plasmid pTrp3.

FIG. 8a shows the HinfI restriction pattern of clone pSY2501 and FIG. 8b shows the resulting two 169bp and 28bp fragments thereof.

FIG. 9 is a restriction map of clone pSY2501.

FIG. 10 shows the coding DNA sequence for the mutein IFN-.beta..sub.ser17 with the corresponding amino acid sequence therefor.

FIG. 11 shows the single 18,000 dalton protein band corresponding to IFN-.beta..sub.ser17 in the extracts of clones pSY2501 and p.beta.1trp.

FIG. 12 is a diagram of the plasmid pLW1 which contains the human interleukin-2 (IL-2) gene under the control of the E .coli trp promoter.

FIG. 13 is a restriction map of phage clone M13-IL2.

FIG. 14 is a restriction map of the plasmid pLW46.

FIGS. 15a and 15b show, respectively, the nucleotide sequence of the coding strand of the clone pLW46 and the corresponding amino acid sequence of the IL-2 mutein designated IL-2.sub.ser125.

FIG. 16 is a diagram of the plasmid pLW32.

FIG. 17 is a diagram of the plasmid pLW55.

#### MODES FOR CARRYING OUT THE INVENTION

The present invention provides: muteins of biologically active proteins in which cysteine residues that are not essential to biological activity have been deliberately deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation; mutant genes coding for such muteins; and means for making such muteins.

Proteins that may be mutationally altered according to this invention may be identified from available information regarding the cysteine content of biologically active proteins and the roles played by the cysteine residues with respect to activity and tertiary structure. For proteins for which such information is not available in the literature this information may be determined by systematically altering each of the cysteine residues of the protein by the procedures described herein and testing the biological activity of the resulting muteins and their proclivity to form undesirable intermolecular or intramolecular disulfide bonds. Accordingly, while the invention is specifically described and exemplified below as regards muteins of IFN-.beta. and IL-2 it will be appreciated that the following teachings apply to any other biologically active protein that contains a functionally nonessential cysteine residue that makes the protein susceptible to undesirable disulfide bond formation. Examples of proteins other than IFN-.beta. and IL-2 that are candidates for mutational alteration according to the invention are lymphotoxin (tumor necrosis factor), colony stimulating factor-1, and IFN-.alpha.1. Candidate proteins will usually have an odd number of cysteine residues.

In the case of IFN-.beta. it has been reported in the literature and that both the glycosylated and unglycosylated IFNs show qualitatively similar specific activities and that, therefore, the glycosyl moieties are not involved in and do not contribute to the biological activity of IFN-.beta.. However, bacterially produced IFN-.beta. which is unglycosylated consistently exhibits quantitatively lower specific activity than native IFN-.beta. which is glycosylated. IFN-.beta. is known to have three cysteine residues at positions 17, 31 and 141. Cysteine 141 has been demonstrated by Shepard, et al, supra, to be essential for biological activity. In IFN-.alpha., which contains four cysteine residues, there are two intramolecular --S--S-- bonds: one between cys 29 and cys 138 and another between cys 1 and cys 98. Based on the homology between IFN-.beta. and IFN-.alpha.s cys 141 of IFN-.beta. could be involved in an intramolecular --S--S-- bond with cys 31, leaving cys 17 free to form intermolecular crosslinks. By either deleting cys 17 or substituting it by a different amino acid, one can determine whether cys 17 is essential to biological activity, and its role in --SS-- bond formation. If cys 17 is not essential for the



biological activity of the protein, the resulting cys 17-deleted or cys 17-substituted protein might exhibit specific activity close to that of native IFN-.beta. and would possibly also facilitate isolation and purification of the protein.

By the use of the oligonucleotide-directed mutagenesis procedure with a synthetic oligonucleotide primer that is complementary to the region of the IFN-.beta. gene at the codon for cys 17 but which contains single or multiple base changes in that codon, a designer gene may be produced that results in cys 17 being replaced with any other amino acid of choice. When deletion is desired the oligonucleotide primer lacks the codon for cys 17. Conversion of cys 17 to neutral amino acids such as glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine is the preferred approach. Serine and threonine are the most preferred replacements because of their chemical analogy to cysteine. When the cysteine is deleted, the mature munein is one amino acid shorter than the native parent protein or the microbially produced IFN-.beta..

Human IL-2 is reported to have three cysteine residues located at positions 58, 105, and 125 of the protein. As in the case of IFN-.beta., IL-2 is in an aggregated oligomeric form when isolated from bacterial cells and has to be reduced with reducing agents in order to obtain a good yield from bacterial extracts. In addition, the purified reduced IL-2 protein is unstable and readily reoxidized upon storage to an oligomeric inactive form. The presence of three cysteines means that upon reoxidation, the protein may randomly form one of three possible intramolecular disulfide bridges, with only one of those being the correct bridge as found in the native molecule. Since the disulfide structure of the native IL-2 protein is not known, it is possible to use the present invention to create mutations at codons 58, 105 and 125 of the IL-2 gene and identify which cysteine residues are necessary for activity and therefore most likely to be involved in native disulfide bridge formation. In the same vein, the cysteine residue that is not necessary for activity can be modified so as to prevent the formation of incorrect intramolecular disulfide bridges and minimize the chance of intermolecular disulfide bridges by replacement of the free cysteine residue.

The size of the oligonucleotide primer is determined by the requirement for stable hybridization of the primer to the region of the gene in which the mutation is to be induced, and by the limitations of the currently available methods for synthesizing oligonucleotides. The factors to be considered in designing oligonucleotides for use in oligonucleotide-directed mutagenesis (eg, overall size, size of portions flanking the mutation site) are described by Smith, M. and Gillam S., supra. In general the overall length of the oligonucleotide will be such as to optimize stable, unique hybridization at the mutation site with the 5' and 3' extensions from the mutation site being of sufficient size to avoid editing of the mutation by the exonuclease activity of the DNA polymerase. Oligonucleotides used for mutagenesis in accordance with the present invention usually contain from about 12 to about 24 bases, preferably from about 14 to about 20 bases and still more preferably from about 15 to about 18 bases. They will usually contain at least about three bases 3' of the altered or missing codon.

The method for preparing the modified IFN-.beta. gene broadly involves inducing a site-specific mutagenesis in the IFN-.beta. gene at codon 17 (TGT) using a synthetic nucleotide primer which omits the codon or alters it so that it codes for another amino acid. When threonine replaces the cysteine and the primer is hybridized to the antisense strand of the IFN-.beta. gene, the preferred nucleotide primer is GCAATTTTCAGACTCAG (underlining denotes the altered codon). When it is desirable to delete cysteine, the preferred primer is AGCAATTTTCAGCAGAAGCTCCTG, which omits the TGT codon for cys. When cysteine is replaced by serine, a 17-nucleotide primer, GCAATTTTCAGAGTCAG, which includes an AGT codon for serine is the primer of choice. The T.fwdarw.A transition of the first base in the cys 17 codon results in changing cysteine to serine. It must be recognized that when deletions are introduced, the proper reading frame for the DNA sequence must be maintained for expression of the desired protein.



The primer is hybridized to single-stranded phage such as M13, fd, or .0.X174 into which a strand of the IFN-.beta. gene has been cloned. It will be appreciated that the phage may carry either the sense strand or antisense strand of the gene. When the phage carries the antisense strand the primer is identical to the region of the sense strand that contains the codon to be mutated except for a mismatch with that codon that defines a deletion of the codon or a triplet that codes for another amino acid. When the phage carries the sense strand the primer is complementary to the region of the sense strand that contains the codon to be mutated except for an appropriate mismatch in the triplet that is paired with the codon to be deleted. Conditions that may be used in the hybridization are described by Smith, M. and Gillam, S., supra. The temperature will usually range between about 0.degree. C. and 70.degree. C., more usually about 10.degree. C. to 50.degree. C. After the hybridization, the primer is extended on the phage DNA by reaction with DNA polymerase I, T.sub.4 DNA polymerase, reverse transcriptase or other suitable DNA polymerase. The resulting dsDNA is converted to closed circular dsDNA by treatment with a DNA ligase such as T.sub.4 DNA ligase. DNA molecules containing single-stranded regions may be destroyed by S1 endonuclease treatment.

Oligonucleotide-directed mutagenesis may be similarly employed to make a mutant IL-2 gene that encodes a mutein having IL-2 activity but having cys 125 changed to serine 125. The preferred oligonucleotide primer used in making this mutant IL-2 gene when the phage carries the sense strand of the gene is GATGATGCTTCTGAGAAAAGGTAATC. This oligonucleotide has a C.fwdarw.G change at the middle base on the triplet that is paired with codon 125 of the IL-2 gene.

The resulting mutational heteroduplex is then used to transform a competent host organism or cell. Replication of the heteroduplex by the host provides progeny from both strands. Following replication the mutant gene may be isolated from progeny of the mutant strand, inserted into an appropriate expression vector, and the vector used to transform a suitable host organism or cell. Preferred vectors are plasmids pBR322, pCR1, and variants thereof, synthetic vectors and the like. Suitable host organisms are E. coli, Pseudomonas, Bacillus subtilis, Bacillus thuringiensis, various strains of yeast, Bacillus thermophilus, animal cells such as mice, rat or Chinese hamster ovary (CHO) cells, plant cells, animal and plant hosts and the like. It must be recognized that when a host of choice is transformed with the vector, appropriate promoter-operator sequences are also introduced in order for the mutein to be expressed. Hosts may be prokaryotic or eukaryotic (processes for inserting DNA into eukaryotic cells are described in PCT applications Nos. US81/00239 and US81/00240 published Sept. 3, 1981). E. coli and CHO cells are the preferred hosts. The muteins obtained in accordance with the present invention may be glycosylated or unglycosylated depending on the glycosylation occurring in the native parent protein and the host organism used to produce the mutein. If desired, unglycosylated mutein obtained when E. coli or a Bacillus is the host organism, may be optionally glycosylated in vitro by chemical, enzymatic and other types of modifications known in the art.

In the preferred embodiment of the subject invention respecting IFN-.beta., the cysteine residue at position 17 in the amino acid sequence of IFN-.beta., as shown in FIG. 1, is changed to serine by a T.fwdarw.A transition of the first base of codon 17 of the sense strand of the DNA sequence which codes for the mature IFN-.beta.. The site-specific mutagenesis is induced using a synthetic 17-nucleotide primer GCAATTTTCAGAGTCAG which is identical to a seventeen nucleotide sequence on the sense strand of IFN-.beta. in the region of codon 17 except for a single base mismatch at the first base of codon 17. The mismatch is at nucleotide 12 in the primer. It must be recognized that the genetic code is degenerate and that many of the amino acids may be encoded by more than one codon. The base code for serine, for example, is six-way degenerate such that the codons, TCT, TCG, TCC, TCA, AGT, and ACG all code for serine. The AGT codon was chosen for the preferred embodiment for convenience. Similarly, threonine is encoded by any one of codons ACT, ACA, ACC and ACG. It is intended that when one codon is specified for a particular amino acid, it includes all degenerate codons which encode

that amino acid. The 17-mer is hybridized to single-stranded M13 phage DNA which carries the antisense strand of the IFN-.beta. gene. The oligonucleotide primer is then extended on the DNA using DNA polymerase I Klenow fragment and the resulting dsDNA is converted to closed circular DNA with T.sub.4 ligase. Replication of the resulting mutational heteroduplex yields clones from the DNA strand containing the mismatch. Mutant clones may be identified and screened by the appearance or disappearance of specific restriction sites, antibiotic resistance or sensitivity, or by other methods known in the art. When cysteine is substituted with serine, the T.fwdarw.A transition, shown in FIG. 2, results in the creation of a new *Hinf*I restriction site in the structural gene. The mutant clone is identified by using the oligonucleotide primer as a probe in a hybridization screening of the mutated phage plaques. The primer will have a single mismatch when hybridized to the parent but will have a perfect match when hybridized to the mutated phage DNA, as indicated in FIG. 2. Hybridization conditions can then be devised where the oligonucleotide primer will preferentially hybridize to the mutated DNA but not to the parent DNA. The newly generated *Hinf*I site also serves as a means of confirming the single base mutation in the IFN-.beta. gene.

The M13 phage DNA carrying the mutated gene is isolated and spliced into an appropriate expression vector, such as plasmid pTrp3, and *E. coli* strain MM294 is transformed with the vector. Suitable growth media for culturing the transformants and their progeny are known to those skilled in the art. The expressed mutein of IFN-.beta. is isolated, purified and characterized.

The following examples are presented to help in the better understanding of the subject invention and for purposes of illustration only. They are not to be construed as limiting the scope of the invention in any manner. Examples 1-11 describe the preparation of a mutein of IFN-.beta.. Examples 12-20 describe the preparation of a mutein of IL-2.

#### EXAMPLE 1

##### Cloning of the IFN-.beta. Gene Into M13 Vector

The use of M13 phage vector as a source of single-stranded DNA template has been demonstrated by G. F. Temple et al, *Nature* (1982) 296: 537-540. Plasmid p.beta.1trp (FIG. 3) containing the IFN-.beta. gene under control of *E. coli* trp promoter, was digested with the restriction enzymes *Hind*III and *Xho*II. The M13mp8 (J. Messing, "Third Cleveland Symposium on Macromolecules: Recombinant DNA," Ed. A. Walton, Elsevier Press, 143-153 (1981)) replicative form (RF) DNA (FIG. 4) was digested with restriction enzymes *Hind*III and *Bam*HI, and mixed with the p.beta.1trp DNA which had previously been digested with *Hind*III and *Xho*II. The mixture was then ligated with T.sub.4 DNA ligase and the ligated DNA transformed into competent cells of *E. coli* strain JM 103 and plated on Xgal indicator plates (J. Messing, et al, *Nucleic Acids Res* (1981) 9: 309-321). Plaques containing recombinant phage (white plaques) were picked, inoculated into a fresh culture of JM 103 and minipreps of RF molecules prepared from the infected cells (H. D. Birnboim and J. Doly, *Nucleic Acid Res* (1979) 7: 1513-1523). The RF molecules were digested with various restriction enzymes to identify the clones containing the IFN-.beta. insert. The restriction map of one such clone (M13-.beta.1) is shown in FIG. 5. Single-stranded (ss) phage DNA was prepared from clone M13-.beta.1 to serve as a template for site-specific mutagenesis using a synthetic oligonucleotide.

#### EXAMPLE 2

##### Site-Specific Mutagenesis

Forty picomoles of the synthetic oligonucleotide GCAATTTTCAGAGTCAG (primer) was treated with T.sub.4 kinase in the presence of 0.1 mM adenosine triphosphate (ATP), 50 mM

hydroxymethylaminomethane hydrochloride (Tris-HCl) pH 8.0, 10 mM MgCl.sub.2, 5 mM dithiothreitol (DTT) and 9 units of T.sub.4 kinase, in 50 .mu.l at 37.degree. C. for 1 hr. The kinased primer (12 pmole) was hybridized to 5 .mu.g of ss M13-.beta.1 DNA in 50 .mu.l of a mixture containing 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl.sub.2 and 10 mM .beta.-mercaptoethanol, by heating at 67.degree. C. for 5 min and at 42.degree. C. for 25 min. The annealed mixture was then chilled on ice and then added to 50 .mu.l of a reaction mixture containing 0.5 mM each of deoxynucleoside triphosphate (dNTP), 80 mM Tris-HCl, pH 7.4, 8 mM MgCl.sub.2, 100 mM NaCl, 9 units of DNA polymerase I, Klenow fragment, 0.5 mM ATP and 2 units of T.sub.4 DNA ligase, incubated at 37.degree. C. for 3 hr and at 25.degree. C. for 2 hr. The reaction was then terminated by phenol extraction and ethanol precipitation. The DNA was dissolved in 10 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50% sucrose and 0.05% bromophenylblue and electrophoresed on 0.8% agarose gel in the presence of 2 .mu.g/ml of ethidium bromide. The DNA bands corresponding to the RF forms of M13-.beta.1 were eluted from gel slices by the perchlorate method (R. W. Davis, et al, "Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, N.Y., p. 178-179 (1980)). The eluted DNA was used to transform competent JM 103 cells, grown overnight and ssDNA isolated from the culture supernatant. This ssDNA was used as a template in a second cycle of primer extension, the gel purified RF forms of the DNA were transformed into competent JM 103 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

### EXAMPLE 3

#### Site Specific Mutagenesis

The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is GCAATTTTCAGACTCAG to change codon 17 of the IFN-.beta. gene from one that codes for cysteine to one that codes for threonine.

### EXAMPLE 4

#### Site Specific Deletion

The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is AGCAATTTTCAGCAGAAGCTCCTG to delete codon 17 of the IFN-.beta. gene.

### EXAMPLE 5

#### Screening And Identification of Mutagenized Plaques

Plates containing mutated M13-.beta.1 plaques (Example 1) as well as two plates containing unmutated M13-.beta.1 phage plaques, were chilled to 4.degree. C. and plaques from each plate transferred onto two nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2N NaOH, 1.5M NaCl and 0.2% Triton X-100 for 5 min, and neutralized by layering onto filter papers soaked with 0.5M Tris-HCl, pH 7.5 and 1.5M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2.times.SSC (standard saline citrate), dried and then baked in a vacuum oven at 80.degree. C. for 2 hr. The duplicate filters were prehybridized at 55.degree. C. for 4 hr with 10 ml per filter of DNA hybridization buffer (5.times.SSC) pH 7.0, 4.times.Denhardt's solution (polyvinylpyrrolidone, ficoll and bovine serum albumin, 1.times.=0.02% of each), 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate buffer pH 7.0 and 100 .mu.g/ml of denatured salmon sperm DNA. .sup.32 P-labeled probe was prepared by kinasing the oligonucleotide primer with .sup.32 P-labeled ATP. The filters were hybridized to 3.5.times.10.sup.5 cpm/ml of .sup.32 P-labeled primer in 5

ml per filter of DNA hybridization buffer at 55.degree. C. for 24 hr. The filters were washed at 55.degree. C. for 30 min each in washing buffers containing 0.1% SDS and decreasing amounts of SSC. The filters were washed initially with buffer containing 2.times.SSC and the control filters containing unmutated M13-.beta.1 plaques were checked for the presence of any radioactivity using a Geiger counter. The concentration of SSC was lowered stepwise and the filters washed until no detectable radioactivity remained on the control filters with the unmutated M13-.beta.1 plaques. The lowest concentration of SSC used was 0.1.times.SSC. The filters were air dried and autoradiographed at -70.degree. C. for 2-3 days. 480 plaques of mutated M13-.beta.1 and 100 unmutated control plaques were screened with the kinased oligonucleotide probe. None of the control plaques hybridized with the probe while 5 mutated M13-.beta.1 plaques hybridized with the probe.

One of the five mutated M13-.beta.1 plaques (M13-SY2501) was picked and inoculated into a culture of JM 103. ssDNA was prepared from the supernatant and double-stranded (ds) DNA was prepared from the cell pellet. The ssDNA was used as a template for the dideoxy-sequencing of the clone using the M13 universal primer. The result of the sequence analysis is shown in FIG. 6, confirming that the TGT cys codon has been converted to an AGT ser codon.

### EXAMPLE 6

#### Expression of Mutated IFN-.beta. in E. coli

RF DNA from M13-SY2501 was digested with restriction enzymes HindIII and XhoII and the 520 bp insert fragment purified on a 1% agarose gel. The plasmid pTrp3 containing the E. coli trp promoter (FIG. 7) was digested with the enzymes HindIII and BamHI, mixed with the purified M13-SY2501 DNA fragment, and ligated in the presence of T.sub.4 DNA ligase. The ligated DNA was transformed into E. coli strain MM294. Ampicillin resistant transformants were screened for sensitivity to the drug tetracycline. Plasmid DNA from five ampicillin resistant, tetracycline sensitive clones were digested with HinfI to screen for the presence of the M13-SY2501 insert. FIG. 8a shows the HINfI restriction pattern of one of the clones (pSY2501), comparing it with the HinfI pattern of the original IFN-.beta. clone, p.beta.1trp. As expected, there is an additional HinfI site in pSY2501, cleaving the 197 bp IFN-.beta. internal fragment to a 169 bp fragment and a 28 fragment (FIG. 8b). A restriction map of the clone pSY2501 is shown in FIG. 9. The complete DNA sequence of the mutant IFN-.beta. gene is shown in FIG. 10 together with the predicted amino acid sequence.

The plasmid designated as clone pSY2501 was deposited with the Agricultural Research Culture Collection (NRRL), Fermentation Laboratory, Northern Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, 1815 North University Street, Peoria, Ill. 60604 on Mar. 30, 1983 and was assigned accession numbers CMCC No. 1533 and NRRL No. B-15356.

Cultures of pSY2501 and p.beta.1trp, which include progeny thereof, were grown up to an optical density (OD.sub.600) of 1.0. Cell free extracts were prepared and the amount of IFN-.beta. antiviral activity assayed on GM2767 cells in a microtiter assay. Extracts of clone pSY2501 exhibited three to ten times higher activity than p.beta.1trp (Table I), indicating that clone pSY2501 was either synthesizing more protein exhibiting IFN-.beta. activity or that the protein made had a higher specific activity.

TABLE I	EXTRACT ANTIVIRAL ACTIVITY (U/ml)
pSY2501	6 .times. 10.sup.5
p.beta.1trp	1 .times. 10.sup.5
ptpr3 (control)	30

In order to determine if clone pSY2501 was synthesizing several times more active protein, the extracts



of both clones were electrophoresed on a SDS polyacrylamide gel together with a control extract and the gel stained with coomassie blue to visualize the proteins. As shown in FIG. 11, there was only one protein band corresponding to an apparent 18,000 dalton protein that was present in the extracts of clones pSY2501 and p.beta.1trp but not in the control extract of ptrp3. This protein, which has a molecular weight of about 20,000 daltons but shows a gel migration pattern of an 18,000 dalton protein was previously shown to be IFN-.beta. by purification of this protein from extracts of p.beta.1trp. Since there is less of this protein in extracts of pSY2501 than in extracts of p.beta.1trp, the specific activity of the protein in extracts of clone pSY2501 was higher than that of clone p.beta.1trp.

#### EXAMPLE 7

The plasmid pSY2501 was transformed into a competent subvariant of E. coli strain MM294, designated MM294-1. A sample of the resulting transformant was deposited in the American Type Culture Collection 12301 Parklawn Drive, Rockville, Md. 20852 USA on Nov. 18, 1983 under ATCC number 39,517.

#### EXAMPLE 8

##### Production of IFN-.beta..sub.ser17

IFN-.beta..sub.ser17 was recovered from E. coli that had been transformed to produce IFN-.beta..sub.ser17. The E. coli were grown in the following growth medium to an OD of 10-11 at 680 nm (dry wt 8.4 g/l).

Ingredient Concentration	
NH.sub.4 Cl	20 mM
K.sub.2 SO.sub.4	16.1 mM
KH.sub.2 PO.sub.4	7.8 mM
Na.sub.2 HPO.sub.4	12.2 mM
MgSO.sub.4 .7H.sub.2 O	3 mM
Na.sub.3 citrate.2H.sub.2 O	1.5 mM
MnSO.sub.4 .4H.sub.2 O	30 .mu.M
ZnSO.sub.4 .7H.sub.2 O	30 .mu.M
CuSO.sub.4 .5H.sub.2 O	3 .mu.M
L-Tryptophan	70 mg/l
FeSO.sub.4 .7H.sub.2 O	72 .mu.M
thiamine.HCl	20 mg/l
glucose	40 g/l
15 pH control with	
NH.sub.4 OH	

A 9.9 l (9.9 kg) harvest of the transformed E. coli was cooled to 20.degree. C. and concentrated by passing the harvest through a cross-flow filter at an average pressure drop of .about.110 kpa and steady-state filtrate flow rate of 260 ml/min until the filtrate weight was 8.8 kg. The concentrate (approximately one liter) was drained into a vessel and cooled to 15.degree. C. The cells in the concentrate were then disrupted by passing the concentrate through a Manton-Gaulin homogenizer at 5.degree. C., .about.69,000 kpa. The homogenizer was washed with one liter phosphate buffered saline, pH 7.4 (PBS), and the wash was added to the disruptate to give a final volume of two liters. This volume was continuously centrifuged at 12000.times.g at a 50 ml/min flow rate. The solid was separated from the supernatant and resuspended in four liters PBS containing 2% by wt SDS. This suspension was stirred at room temperature for 15 min after which there was no visible suspended material. The solution was then extracted with 2-butanol at a 1:1 2-butanol:solution volume ratio. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic phase was then separated and evaporated to dryness to yield 21.3 g of protein. This was resuspended in distilled water at a 1:10 volume ratio.

The recovered product was assayed for human IFN-.beta. activity using an assay based on protection against viral cytopathic effect (CPE). The assay was made in microtiter plates. Fifty .mu.l of minimum essential medium were charged into each well and 25 .mu.l of the sample was placed in the first well and 1:3 volume dilutions were made serially into the following wells. Virus (vesicular stomatitis), cell

(human fibroblast line GM-2767), and reference IFN-.beta. controls were included on each plate. The reference IFN-.beta. used was 100 units per mol. The plates were then irradiated with UV light for 10 min. After irradiation 100 .mu.l of the cell suspension ( $1.2 \times 10^5$  cells/ml) was added to each well and the trays were incubated for 18-24 hr. A virus solution at one plaque-forming unit per cell was added to each well except the cell control. The trays were then incubated until the virus control showed 100% CPE. This normally occurred 18-24 hr after adding the virus solution. Assay results were interpreted in relation to the location of the 50% CPE well of the reference IFN-.beta. control. From this point the titer of interferon for all samples on the plate was determined. The specific activity of the recovered product was determined to be  $5 \times 10^7$  U/mg.

#### EXAMPLE 9

##### Acid Precipitation And Chromatographic Purification

The process of Example 8 was repeated except that after extraction and separation of the aqueous and organic phases and mixing of the organic phase with PBS at a volume ratio of 3:1 the pH of the mixture was lowered to about 5 by addition of glacial acetic acid. The resulting precipitate was separated by centrifugation at 10,000-17,000.times.g for 15 min and the pellet was redissolved in 10% w/v SDS, 10 mM DTT, 50 mM sodium acetate buffer, pH 5.5, and heated to 80.degree. C. for 5 min.

The solution was then applied to a Brownlee RP-300, 10 .mu.M, "Aquapore" column using a Beckman gradient system. Buffer A was 0.1% trifluoroacetic acid (TFA) in H.sub.2 O; buffer B was 0.1% TFA in acetonitrile. Detection was by ultraviolet absorbance at 280 nm. The solvent program was linear gradient of 0% buffer B to 100% buffer B in three hr. Fractions containing highest interferon activities were pooled and the specific activity of the pooled interferon preparation was determined to be  $9.0 \times 10^7$  to  $3.8 \times 10^8$  international units per mg protein, as compared to about  $2 \times 10^8$  U/mg for native IFN-.beta..

#### EXAMPLE 10

##### Biochemical Characterization of IFN-.beta. Ser.sub.17

Amino acid compositions were determined after 24-72 hr timed hydrolysis of 40 .mu.g samples of IFN in 200 .mu.l of 5.7N HCl, 0.1% phenol, at 108.degree. C. Proline and cysteine were determined in the same fashion after performic acid oxidation; in this case, phenol was omitted from the hydrolysis. Tryptophan was analyzed after 24 hr hydrolysis of 400 .mu.l samples in 5.7N HCl, 10% mercaptoacetic acid (no phenol). Analysis was performed on a Beckman 121MB amino acid analyzer using a single column of AA10 resin.

The amino acid composition calculated from representative 24-, 48-, 72-hr acid hydrolyses of purified IFN-.beta. Ser.sub.17 agrees well with that predicted by the DNA sequence of the cloned IFN gene, minus the missing N-terminal methionine.

The amino acid sequence of the first 58 residues from the amino acid terminus of purified IFN was determined on a 0.7 mg sample in a Beckman 890C sequanator with 0.1M Quadrol buffer. PTH amino acids were determined by reverse-phase HPLC on an Altex ultrasphere ODS column ( $4.6 \times 250$  mm) at 45.degree. C. eluted at 1.3 min at 40% buffer B, and 8.4 min from 40-70% buffer B, where buffer A was 0.0115M sodium acetate, 5% tetrahydrofuran (THF), pH 5.11 and buffer B was 10% THF in acetonitrile.

The N-terminal amino acid sequence of IFN-.beta. Ser.sub.17 determined matches the expected



sequence predicted from the DNA sequence, except for the absence of N-terminal methionine.

#### EXAMPLE 11

##### Alternative IFN- $\beta$ Production and Purification Process

*E. coli* transformed with pSY2501 were grown in the following medium:

	Approximate Initial Ingredient Concentration
30 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 74 mM MgSO <sub>4</sub> ·7H <sub>2</sub> O 3 mM MnSO <sub>4</sub> ·H <sub>2</sub> O 46 $\mu$ M ZnSO <sub>4</sub> ·7H <sub>2</sub> O 46 $\mu$ M CuSO <sub>4</sub> ·5H <sub>2</sub> O 1-2 $\mu$ M L-tryptophan 350 $\mu$ M FeSO <sub>4</sub> ·7H <sub>2</sub> O 74 $\mu$ M thiamine.HCl 0.002% glucose 0.5%	Na <sub>3</sub> Citrate 2 O 3 mM KH <sub>2</sub> PO <sub>4</sub>

Dow Corning Antifoam polypropylene glycol, 25% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

Temperature was maintained at 37. $\pm$ 1. $^{\circ}$ C., pH at 6.5. $\pm$ 0.1 with NaOH, and dissolved oxygen at 30% of air saturation. Optical density and residual glucose measurements were taken at 14 hr and at approximately one hr intervals thereafter. Harvest was made when glucose consumption reached 40. $\pm$ 0.6 g/l (OD at 680 nm=10-11).

The harvested material was concentrated approximately 3-fold by circulating it through a microporous cross-flow filter under pressure. The concentrated cells were diafiltered against deionized water until the harvest material was concentrated 4-5 fold. The cells were then disrupted by passing them through a Manton-Gaulion homogenizer at about 4.1-5.5 times 10<sup>4</sup> kpa. After the initial pass SDS-sodium phosphate buffer was added to a final concentration of 2% SDS, 0.08M sodium phosphate and homogenization was continued for one hr. Solid DTT was then added to a final concentration of 50 mM and the homogenizate was heated to 90. $\pm$ 0.5. $^{\circ}$ C. for 10 min. The resulting cell suspension was extracted with 2-butanol at a 1:1 2-butanol:suspension volume ratio in a static mixer. The mixture was then centrifuged and the 2-butanol rich phase was collected.

The 2-butanol rich phase was mixed with 2.5 volumes of 0.1% SDS in PBS. Solid DTT was added to a final concentration of 2 mM. The pH of the mixture was adjusted to 6.2. $\pm$ 0.1 with glacial acetic acid and this mixture was centrifuged. The resulting paste was collected and resuspended in PBS +10% SDS with pH adjustment to 8.5. $\pm$ 0.1 using 1N NaOH. Solid DTT was added to a final concentration of 100 mM and the suspension was heated to 90. $\pm$ 0.5. $^{\circ}$ C. for 10 min. The suspension was then cooled to about 25. $^{\circ}$ C., the pH was adjusted to 5.5. $\pm$ 0.1 with glacial acetic acid, and the solution was filtered.

The solution was then applied to a Sephacryl S-200 pre column and the fractions containing highest interferon activities were pooled and concentrated by ultrafiltration with a 10 Kdal molecular weight cutoff. The concentrate was oxidized by adding equimolar amounts of protein and iodosobenzoic acid into a reaction vessel containing 2 mM sodium pyrophosphate, 0.1% SDS and 1 mM EDTA. The pH was controlled during oxidation at 9.0. $\pm$ 0.1 with 0.5N NaOH and adjusted to 5.5. $\pm$ 0.2 when oxidation was complete. After oxidation the concentrate was again passed through the ultrafiltration unit with a 10 Kdal molecular weight cutoff.

The concentrate was applied to a main Sephacryl S-200 column and the fractions were analyzed by SDS-PAGE to determine those containing no high molecular weight contaminants. Those fractions were

pooled and passed through the ultrafiltration unit. The filtered concentrate was then fractionated on a Sephadex G-75 column. SDS-PAGE analysis of the fractions was made to determine those containing no low or high molecular weight contaminants. Those fractions were pooled for desalting.

A Sephadex G-25 column equilibrated with 1 mM NaOH was loaded with the pooled fractions from the Sephadex G-75 column using distilled water adjusted to pH 10.8-11 with 50% NaOH. The purified product was collected as the void volume peak. This desalted, purified IFN-.beta. mutein may be formulated in known manners for therapeutic administration.

#### Biological Testing of IFN-.beta..sub.ser17

##### Antigenic Comparison

IFN-.beta..sub.ser17 was compared antigenically to IFN-.beta. produced from diploid fibroblasts using virus neutralizing tests. A polyvalent antiserum to the diploid fibroblast IFN-.beta. was prepared in rabbits. This antiserum blocked the antiviral activity of both the diploid fibroblast IFN-.beta. and the IFN-.beta..sub.ser17 in the virus neutralization tests, indicating the two proteins are indistinguishable antigenically.

##### Antiviral Activity

The purified IFN-.beta..sub.ser17 was compared in its antiviral activity to naturally produced IFN-.beta.. Inhibition of vesicular stomatitis virus replication in diploid foreskin fibroblast (HS27F) was indistinguishable from that of the natural molecule. Similarly, inhibition of herpes simplex virus type 1 in HS27F fibroblasts by the natural and mutant proteins were comparable.

##### Antiproliferative Activity

The antiproliferation activity of IFN-.beta..sub.ser17 for continuous cell lines was compared with that of naturally produced IFN-.beta.. T24 cells derived from a transitional cell carcinoma were treated with 200 units/ml of the proteins. Cell growth was inhibited significantly ( $p < 0.02$ ) by both proteins.

##### Natural Killer (NK) Cell Stimulation

The ability of IFN-.beta..sub.ser17 to stimulate NK cell (spontaneous cell mediated cytotoxicity) activity was tested. Ficoll-hypaque separated peripheral human mononuclear cells (PMC) or NK-enriched lymphocyte preparations (depleted of monocytes by plastic adherence and of OKT3-positive T cells by treatment with OKT3 antibody plus complement) were incubated overnight in growth medium containing various concentrations of IFN-.beta..sub.ser17. <sup>51</sup>Cr-labeled target cells were incubated with the effector cells (effector cell:target cell ratio=50:1) for 2-4 hours. NK cell cytotoxicity was determined by measuring the amount of label released into the medium. The results of these tests are reported in Table I below.

TABLE I

NK Cell Cytotoxicity by Interferon (specific % <sup>51</sup>Cr release  $\pm$  SEM) IFN (units/ml) Target  
Effector Cell Cells 0 10 30 100 300 1000

						T24 PMC	
7.23	$\pm$ 5.1	23.1	$\pm$ 4.4	24.4	$\pm$ 1.1	34.1	$\pm$ 2.5
50.0	$\pm$ 2.0	40.4	$\pm$ 4.4	Chang PMC	4.7	$\pm$ 0.5	
7.2	$\pm$ 0.8	9.5	$\pm$ 1.7	15.9	$\pm$ 1.3	21.9	$\pm$ 1.4
26.9	$\pm$ 1.8	Chang NK Enr	19.2	$\pm$ 4.6	39.4	$\pm$ 4.1	
ND 54.2	$\pm$ 6.1	ND 41.7	$\pm$ 5.5	K562 NK Enr	41.0	$\pm$ 4.6	48.4
$\pm$ 3.5							$\pm$ 3.6
							ND 62.2
							$\pm$ 3.5
							ND 63.2
							$\pm$ 3.5

As shown the target cells were killed more effectively by the IFN-.beta..sub.ser17 -treated cells than by the untreated cells.

## Clinical Trials

Phase I clinical trials to verify the safety of IFN-.beta..sub.ser17 in humans have been initiated. These trials involve administering the protein to patients intramuscularly and intravenously at doses ranging between 1.times.10.sup.5 units (1 .mu.g of protein) to 400.times.10.sup.6 units. In initial phase I clinical trials no unexpected adverse effects have occurred.

As indicated above, the IFN-.beta..sub.ser17 preparation exhibits specific activity levels very close to or better than that of native IFN-.beta.. IFN-.beta..sub.ser17 has no free sulfhydryl groups but indicates one --S--S-- bond between the only remaining cysteines at positions 31 and 141. The protein does not readily form oligomers and appears to be substantially in the monomeric form. The IFN-.beta..sub.ser17 obtained in accordance with this invention may be formulated either as a single product or mixtures of the various forms, into pharmaceutically acceptable preparations in inert, nontoxic, nonallergenic, physiologically compatible carrier media for clinical and therapeutic uses in cancer therapy or in conditions where interferon therapy is indicated and for viral infections such as herpes simplex virus I and II, hepatitis B virus, common cold viruses, and rhinovirus. Such media include but are not limited to distilled water, physiological saline, Ringer's solution, Hank's solution and the like. Other nontoxic stabilizing and solubilizing additives such as dextrose, HSA (human serum albumin) and the like may be optionally included. The therapeutic formulations may be administered orally or parenterally such as intravenous, intramuscular, intraperitoneal and subcutaneous administrations. Preparations of the modified IFN-.beta. of the present invention may also be used for topical application in appropriate media normally utilized for such purposes. The IFN-.beta. mutein may be administered either locally or systemically by itself or in combination or conjunction with other therapeutic agents such as acyclovir for prophylactic or therapeutic purposes for example, U.S. Pat. No. 4,355,032. The dose of mutein administered to human patients will depend on whether it is administered continuously (including intermittent) or as a bolus. The amounts administered continuously will typically be lower than the amounts administered as a bolus. The amount will usually be in the range of about 1.times.5.sup.4 to 4.times.10.sup.8 units, more usually about 1.times.10.sup.6 to 1.times.10.sup.7 units.

The principal advantages of the above described mutein of IFN-.beta. lie in the elimination of a free sulfhydryl group at position 17 in IFN-.beta., thereby forcing the protein to form correct disulfide links between cys 31 and cys 141 and to assume the conformation ostensibly required for full biological activity. The increased specific activity of the IFN-.beta..sub.ser17 enables the use of smaller dosages in therapeutic uses. By deleting the free --SH group, the IFN-.beta..sub.ser17 protein does not form dimers and oligomers so readily as the microbially produced IFN-.beta.. This facilitates purification of the protein and enhances its stability.

## EXAMPLE 12

The nucleotide sequence for a cDNA clone coding for human IL-2, procedures for preparing IL-2 cDNA libraries, and screening same for IL-2 are described by Taniguchi, T., et al, Nature (1983) Vol 24, p 305 et seq.

cDNA libraries enriched in potential IL-2 cDNA clones were made from an IL-2 enriched mRNA fraction obtained from induced peripheral blood lymphocytes (PBL) and Jurkat cells by conventional procedures. The enrichment of the mRNA for IL-2 message was made by fractionating the mRNA and identifying the fraction having IL-2 mRNA activity by injecting the fractions in *Xenopus laevis* oocytes

and assaying the oocyte lysates for IL-2 activity on HT-2 cells (J. Watson, J. Exp Med (1979) 150: 1570-1519 and S. Gillis et al, J Immun (1978) 120: 2027-2032.)

#### EXAMPLE 13

##### Screening and Identification of IL-2 cDNA Clones

The IL-2 cDNA libraries were screened using the colony hybridization procedure. Each microtiter plate was replicated onto duplicate nitrocellulose filter papers (S & S type BA-85) and colonies were allowed to grow at 37.degree. C. for 14-16 hr on L agar containing 50 .mu.g/ml ampicillin. The colonies were lysed and DNA fixed to the filter by sequential treatment for 5 min with 500 mM NaOH, 1.5M NaCl, washed twice for 5 min each time with 5.times.standard saline citrate (SSC). Filters were air dried and baked at 80.degree. C. for 2 hr. The duplicate filters were pre-hybridized at 42.degree. C. for 6-8 hr with 10 ml per filter of DNA hybridization buffer (50% formamide, 5.times.SSC, pH 7.0, 5.times.Denhardt's solution (polyvinylpyrrolidone, plus ficoll and bovine serum albumin; 1.times.=0.2% of each), 50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 20 .mu.g/ml Poly U, and 50 .mu.g/ml denatured salmon sperm DNA.

A .sup.32 P-labeled 20-mer oligonucleotide probe was prepared based on the IL-2 gene sequence reported by Taniguchi, T., et al, supra. The nucleotide sequence of the probe was GTGGCCTTCTTGGGCATGTA.

The samples were hybridized at 42.degree. C. for 24-36 hr with 5 ml/filter of DNA hybridization buffer containing the .sup.32 P nucleotide probe. The filters were washed two times for 30 min each time at 50.degree. C. with 2.times.SSC, 0.1% SDS, then washed twice with 1.times.SSC and 0.1% SDS at 50.degree. C. for 90 min, air dried, and autoradiographed at -70.degree. C. for 2 to 3 days. Positive clones were identified and rescreened with the probe. Full length clones were identified and confirmed by restriction enzyme mapping and comparison with the sequence of the IL-2 cDNA clone reported by Taniguchi, T., et al, supra.

#### EXAMPLE 14

##### Cloning of IL-2 Gene into M13 Vector

The IL-2 gene was cloned into M13mp9 as described in Example 1 using the plasmid pLW1 (FIG. 12) containing the IL-2 gene under the control of the E. coli trp promoter. A sample of pLW1 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, USA, on 4 Aug. 1983 and has been assigned ATCC number 39,405. The restriction map of one clone (designated M13-IL2) containing the IL-2 insert is shown in FIG. 13. Single-stranded phage DNA was prepared from clone M13-IL2 to serve as a template for oligonucleotide-directed mutagenesis.

#### EXAMPLE 15

##### Oligonucleotide-directed Mutagenesis

As indicated previously, IL-2 contains cysteine residues at amino acid positions 58, 105 and 125. Based on the nucleotide sequences of the portions of the IL-2 gene that contain the codons for these three cysteine residues three oligonucleotide primers were designed and synthesized for mutating the codons for these residues to codons for serine. These oligonucleotides have the following sequences.

CTTCTAGAGACTGCAGATGTTTC (DM27) to change cys 58,



CATCAGCATACTCAGACATGAATG (DM28) to change cys 105 and

GATGATGCTCTGAGAAAAGGTAATC (DM29) to change cys 125.

Forty picomoles of each oligonucleotide were kinased separately in the presence of 0.1 mM ATP, 50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mM DTT and 9 units of T<sub>4</sub> kinase in 50  $\mu$ l at 37.degree. C. for 1 hr. Each of the kinased primers (10 pmoles) was hybridized to 2.6  $\mu$ g of ss M13-IL2 DNA in 15  $\mu$ l of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM MgCl<sub>2</sub> and 20 mM  $\beta$ -mercaptoethanol, by heating at 67.degree. C. for 5 min and 42.degree. C. for 25 min. The annealed mixtures were chilled on ice and then adjusted to a final volume of 25  $\mu$ l of a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM MgCl<sub>2</sub>, 83 mM NaCl, 17 mM  $\beta$ -mercaptoethanol, 5 units of DNA polymerase I Klenow fragment, 0.5 mM ATP and 2 units of T<sub>4</sub> DNA ligase, incubated at 37.degree. C. for 5 hr. The reactions were terminated by heating to 80.degree. C. and the reaction mixtures used to transform competent JM103 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

#### EXAMPLE 16

##### Screening and Identification of Mutagenized Phage Plaques

Plates containing mutagenized M13-IL2 plaques as well as 2 plates containing unmutagenized M13-IL2 phage plaques, were chilled to 4.degree. C. and phage plaques from each plate were transferred onto two nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2N NaOH, 1.5M NaCl and 0.2% Triton for 5 min, and neutralized by layering onto filter papers soaked with 0.5M Tris-HCl, pH 7.5, and 1.5M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2.times.SSC, dried and then baked in a vacuum oven at 80.degree. C. for 2 hr. The duplicate filters were pre-hybridized at 42.degree. C. for 4 hr with 10 ml per filter of DNA hybridization buffer (5.times.SSC, pH 7.0, 4.times.Denhardt's solution (polyvinylpyrrolidone, ficoll and bovine serum albumin, 1.times.=0.02% of each), 0.1% SDS, 50 mM sodium phosphate buffer, pH 7.0 and 100  $\mu$ g/ml of denatured salmon sperm DNA. <sup>32</sup>P-labelled probes were prepared by kinasing the oligonucleotide primers with labelled ATP. The filters were hybridized to 0.1.times.10<sup>5</sup> cpm/ml of <sup>32</sup>P-labelled primers in 5 ml per filter of DNA hybridization buffer at 42.degree. C. for 8 hr. The filters were washed twice at 50.degree. C. for 30 min each in washing buffers containing 0.1% SDS and 2.times.SSC, and twice at 50.degree. C. for 30 min each with 0.1% SDS and 0.2.times.SSC. The filters were air dried and autoradiographed at -70.degree. C. for 2-3 days.

Since the oligonucleotide primers DM28 and DM29 were designed to create a new DdeI restriction site in the mutagenized clones (FIG. 14), RF-DNA from a number of the clones which hybridized with each of these kinased primers were digested with the restriction enzyme DdeI. One of the mutagenized M13-IL2 plaques which hybridized with the primer DM28 and has a new DdeI restriction site (M13-LW44) was picked and inoculated into a culture of JM103, ssDNA was prepared from the culture supernatant and dsRF-DNA was prepared from the cell pellet. Similarly, a plaque which hybridized with primer DM29 was picked (M13-LW46) and ssDNA and RF-DNA prepared from it. The oligonucleotide primer DM27 was designed to create a new PstI restriction site instead of a DdeI site. Therefore, the plaques that hybridized to this primer were screened for the presence of a new PstI site. One such phage plaque was identified (M13-LW42) and ssDNA and RF-DNA prepared from it. The DNA from all three of these clones were sequenced to confirm that the target TGT codons for cysteine had been converted to a TCT codon for serine.

# EXAMPLE 17

## Recloning of the Mutagenized IL-2 Gene for Expression in E. coli

RF-DNA from M13-LW42, M13-LW44 and M13-LW46 were each digested with restriction enzymes HindIII and BanII and the insert fragments purified from a 1% agarose gel. Similarly, the plasmid pTrp3 (FIG. 7) was digested with HindIII and BanII, the large plasmid fragment containing the trp promoter was purified on an agarose gel and then ligated with each of the insert fragments isolated from M13-LW42, M13-LW44 and M13-LW46. The ligated plasmids were transformed into competent E. coli K12 strain MM294. The plasmid DNAs from these transformants were analyzed by restriction enzyme mapping to confirm the presence of the plasmids pLW42, pLW44 and pLW46. FIG. 14 is a restriction map of pLW46. When each of these individual clones were grown in the absence of tryptophane to induce the trp promoter and cell free extracts analyzed on SDS-polyacrylamide gels, all three clones, pLW42, pLW44 and pLW46, were shown to synthesize a 14.5 kd protein similar to that found in the positive control, pLW21, which has been demonstrated to synthesize a 14.4 kd IL-2 protein. When these same extracts were subjected to assay for IL-2 activity on mouse HT-2 cells, only clones pLW21 (positive control) and pLW46 had significant amounts of IL-2 activity (Table II below), indicating that cys 58 and cys 105 are necessary for biological activity and changing them to serines (pLW42 and pLW44 respectively) resulted in the loss of biological activity. Cys 125 on the other hand must not be necessary for biological activity because changing it to ser 125 (pLW46) did not affect the biological activity.

TABLE II		Clones IL-2 Activity (.mu./ml)	
		pIL2-7 (negative control)	1 pLW21 (positive control)
113,000	pLW42 660	pLW44 1,990	pLW46 123,000

FIG. 15a shows the nucleotide sequence of the coding strand of clone pLW46. As compared to the coding strand of the native human IL-2 gene clone pLW46 has a single base change of G.fwdarw.C at nucleotide 374. FIG. 15b shows the corresponding amino acid sequence of the IL-2 mutein encoded by pLW46. This mutein is designated des-alanyl (ala) IL-2.sub.ser125 As compared to native IL-2 the mutein has a serine instead of a cysteine at position 125, has an initial N-terminal methionine (which is processed off), and lacks the initial N-terminal alanine of the native molecule.

A sample of E. coli K12 strain MM294 transformed with pLW46 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, USA on 26 Sept. 1983 and has been assigned ATCC Number 39,452.

Examples 18 and 19 describe the preparation of an alternative and preferred vector for expressing alanyl (ala) IL-2.sub.ser125.

# EXAMPLE 18

## Construction of Ala-IL-2 Expression Vector pLW32

A codon (GCG) for alanine was inserted immediately after the initiation codon of the IL-2 gene of pLW1 by oligonucleotide-directed mutagenesis as follows. The oligonucleotide primer, 5'-GAAGTAGGCGCCATAAG-3', was kinased, hybridized to ssM13-IL2 DNA, and extended using the general procedure of Example 15 to form a mutational heteroduplex. In addition to the insertion of the GCG codon, the mutagenesis generated a new NarI restriction site in the gene. The heteroduplex was converted to closed circular heteroduplex and the circular heteroduplexes were used to transform competent JM103 cells and plated onto agar plates and incubated as in Example 15. The plates were



screened to identify mutagenized M13-IL2 by the procedure of Example 16. One mutagenized phage, identified as M13-LW32, was selected for use in additional cloning and RF-DNA was prepared from it. FIG. 16 is a diagram of plasmid pLW32.

#### EXAMPLE 19

##### Construction of Ala-IL-2.sub.ser125 Expressing Clone pLW55

RF-DNA from M13-LW46 (Examples 16 and 17) was digested with XbaI and PstI and the 530 bp fragment containing the carboxy terminal coding region of the IL-2.sub.ser125 gene was purified from an agarose gel. Similarly, pLW32 was digested with XbaI and PstI and the large fragment consisting of the plasmid vector and the ala-IL-2 N-terminal coding sequence was purified. The two purified DNA fragments were pooled and ligated using T.sub.4 DNA ligase. The ligated DNA was transformed into competent E. coli K-12 strain MM294. Tetracycline resistant transformants were analyzed by restriction enzyme mapping for the presence of a plasmid containing an ala-IL-2.sub.ser125 gene, identified as pLW55, which has a new DdeI site not found in pLW32. FIG. 17 is a diagram of pLW55. Cell free extracts of bacterial culture containing pLW55 were found to contain over 10.sup.5 units of IL-2 activity per ml by the HT-2 cell assay, J. Watson, supra, and S. Gillis, supra. Ala-IL-2.sub.ser124 protein is identical to the IL-2.sub.ser125 molecule shown in FIG. 15(b) except that the former includes the initial N-terminal alanine of the native molecule.

A sample of E. coli K-12 strain MM294 transformed with pLW55 was deposited in the American Type Culture Collection on 18 Nov. 1983 and has been assigned ATCC number 39,516.

#### EXAMPLE 20

##### Ala-IL-2.sub.ser125 Production and Purification

E. coli transformed with pLW55 were grown in a fermenter containing the following medium:

---

(NH.sub.4) 150 mM KH.sub.2 PO.sub.4 21.6 mM  
Na.sub.3 Citrate 1.5 mM ZnSO.sub.4.7H.sub.2 O 30 .mu.M MnSO.sub.4.5H.sub.2 O 30 .mu.M  
CuSO.sub.4 5H.sub.2 O 1 .mu.M pH adjusted to 6.50 with 2.5 N NaOH autoclaved Sterile Additions  
(post autoclave) MgSO.sub.4.7H.sub.2 O 3 mM FeSO.sub.4 100 .mu.M L-tryptophan 14 mg/l  
Thiamine-HCl 20 mg/l Glucose 5 g/l Tetracycline 5 mg/l Ethanol 2% Casamino acids 2%

---

Dow Corning Antifoam polypropylene glycol, 20% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

The pH of the fermenter was maintained at 6.8 with 5N KOH. Residual glucose was maintained between 5-10 g/l, dissolved oxygen at 40%, and temperature at 37.degree.+-1.degree. C. The casamino acids (20% stock solution) to a concentration of 2% were added when the OD.sub.680 was about 10. Harvest was made three hr after the OD reached about 20.

The harvested material was concentrated and homogenized as in Example 11. Following DTT-heat treatment, the material was centrifuged and the resulting paste was extracted with urea to a final concentration of 4M. The suspension was centrifuged and SDS was added to the solid phase to a concentration of 5%.

The solution was applied to a Sephacryl S-200 column and fractions containing IL-2 (by SDS-PAGE)

were pooled. The pooled fractions were applied to a Whatman M-40 column packed with 18 micron Vydac C.sub.4 300 .ANG. pore size bonded phase silica gel equilibrated in 0.1% TFA. The IL-2 mutein was eluted with a gradient of 40% to 60% 2-propanol, containing 0.1% TFA, in 160 min. Fractions containing highest IL-2 activities were pooled and found to have specific activities comparable to native IL-2.

Muteins of IL-2 in which the cysteine at position 125 has been replaced with another amino acid, such as the mutein IL-2.sub.ser125 retain IL-2 activity. They may, therefore, be formulated and used in the same manner as native IL-2. Accordingly, such IL-2 muteins are useful for the diagnosis and treatment (local or systemic) of bacterial, viral, parasitic, protozoan and fungal infections; for augmenting cell-mediated cytotoxicity; for stimulating lymphokine activated killer cell activity; for mediating recovery of immune function of lymphocytes; for augmenting alloantigen responsiveness; for facilitating recovery of immune function in acquired immune deficient states; for reconstitution of normal immunofunction in aged human and animals; in the development of diagnostic assays such as those employing enzyme amplification, radiolabelling, radioimaging, and other methods known in the art for monitoring IL-2 levels in the diseased state; for the promotion of T cell growth in vitro for therapeutic and diagnostic purposes for blocking receptor sites for lymphokines; and in various other therapeutic, diagnostic and research applications. The various therapeutic and diagnostic applications of human IL-2 have been investigated and reported in S. A. Rosenberg, E. A. Grimm, et al, A. Mazumder, et al, and E. A. Grimm and S. A. Rosenberg. IL-2 muteins may be used by themselves or in combination with other immunologically relevant B or T cells or other therapeutic agents. Examples of relevant cells are B or T cells, natural killer cells, and the like and exemplary therapeutic reagents which may be used in combination with the polypeptides of this invention are the various interferons, especially gamma interferon, B cell growth factor, IL-1 and the like. For therapeutic or diagnostic applications, they may be formulated in nontoxic, nonallergenic, physiologically compatible carrier media such as distilled water, Ringer's solution, Hank's solution, physiological saline and the like. Administrations of the IL-2 muteins to humans or animals may be oral or intraperitoneal or intramuscular or subcutaneous as deemed appropriate by the physician. The amount of IL-2 mutein administered will usually range between about 1.times.10.sup.4 and 2.times.10.sup.8 units.

Modifications of the above described modes for carrying out the invention that are obvious to those of skill in the fields of genetic engineering, protein chemistry, medicine, and related fields are intended to be within the scope of the following claims.

\* \* \* \* \*

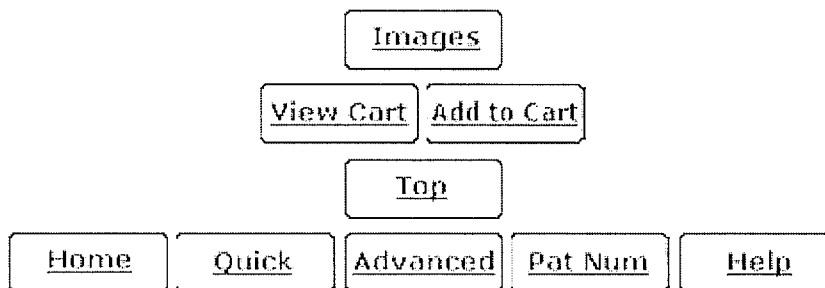


EXHIBIT Q  
PATENT 4,959,314

# USPTO PATENT FULL-TEXT AND IMAGE DATABASE

<a href="#">Home</a>	<a href="#">Quick</a>	<a href="#">Advanced</a>	<a href="#">Pat Num</a>	<a href="#">Help</a>
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<a href="#">Images</a>				

( 1 of 1 )

**United States Patent**  
**Mark , et al.**

**4,959,314**  
**September 25, 1990**

Cysteine-depleted muteins of biologically active proteins

## **Abstract**

Muteins of biologically active proteins such as IFN-.beta. and IL-2 in which cysteine residues that are not essential to biological activity have been deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bridge formation. These muteins are made via bacterial expression of mutant genes that encode the muteins that have been synthesized from the genes for the parent proteins by oligonucleotide-directed mutagenesis.

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[\*] Notice: The portion of the term of this patent subsequent to May 21, 2002 has been disclaimed.

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Filed: **February 7, 1985**

## **Related U.S. Patent Documents**

<u>Application Number</u>	<u>Filing Date</u>	<u>Patent Number</u>	<u>Issue Date</u>
670360	Nov., 1984		
564224	Dec., 1983	4518584	
486162	Apr., 1983		
435154	Oct., 1982		
670360			
661026	Oct., 1984		

**Current U.S. Class:**

**424/85.5 ; 424/85.2; 424/85.6; 435/252.3; 435/252.33;**  
**435/320.1; 435/488; 435/69.1; 435/91.4; 435/91.41;**

435/91.42; 435/91.5; 514/12; 514/2; 514/8; 530/324;  
 530/333; 530/350; 530/351; 536/23.5; 536/23.52  
**Current International Class:** C07K 14/435 (20060101); C07K 14/525 (20060101); A61K  
 38/00 (20060101); C12P 021/00 (); C12P 019/34 (); C12P  
 021/22 ()  
**Field of Search:** 435/68,172.3,253,317,69.1,320,252.3,252.33  
 530/324,333,350,351,828 935/10,27,67,73 514/2,8,12 536/27  
 424/85.2,85.6

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 Zolter et al. Methods in Enzymology, vol. 100, pp. 468-500, 1983, "Oligonocteotide-Directed Mutagenesis of DNA Fragments Cloned Into M13 Vectors" ..

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### Parent Case Text

### CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. Ser. No. 564,224, filed Dec. 20, 1983 now U.S. Pat. No. 4,518,584 which is a continuation-in-part of U.S. Ser. No. 486,162, now abandoned filed Apr. 15, 1983, which is a continuation-in-part of U.S. Ser. No. 435,154 filed Oct. 19, 1982, now abandoned. It is also a continuation-in-part of U.S. Ser. No. 698,939, filed Feb. 7, 1985, now U.S. Pat. No. 4,959,314 which is a continuation-in-part of pending U.S. Ser. No. 670,360, filed Nov. 9, 1984, which is a continuation-in-part of U.S. Ser. No. 661,026, filed Oct. 15, 1984, now abandoned.

### Claims

We claim:

1. A synthetic mutein of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutein having at least one of said cysteine residues substituted by another amino acid and said mutein exhibiting the biological activity of said native protein.
2. The synthetic mutein of claim 1 wherein there is only one of said cysteine residues comprised in the

biologically active native proteins.

3. The synthetic mutein of claim 1 wherein said cysteine residues are replaced by serine, threonine, glycine, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, tryptophan, or methionine.
4. The synthetic mutein of claim 1 wherein said cysteine residues are replaced by serine or threonine.
5. The synthetic mutein of claim 1 wherein the mutein is unglycosylated.
6. A therapeutic formulation comprising an effective amount of the mutein of claims 1, 2, 3, 4 or 5, and at least one other anti-cancer or anti-viral compound.
7. The formulation of claim 6 wherein the anti-cancer or anti-viral compound is gamma interferon.
8. A structural gene having a DNA sequence that encodes a synthetic mutein of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said native mutein having at least one of said cysteine residues substituted by another amino acid and said mutein exhibiting the biological activity of said native protein.
9. A structural gene having a DNA sequence that encodes the synthetic mutein of claim 8 wherein there is only one of said cysteine residues comprised in the biologically active native protein.
10. A structural gene having a DNA sequence that encodes the synthetic mutein of claim 8 wherein said cysteine residues are substituted by serine, threonine, glycine, alanine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan or methionine.
11. A structural gene having a DNA sequence that encodes the synthetic mutein of claim 8 wherein said cysteine residues are substituted by serine or threonine.
12. A structural gene having a DNA sequence that encodes the synthetic mutein of claim 8 wherein the mutein is unglycosylated.
13. An expression vector that includes the structural gene of claim 1, 9, 10, 11 or 12 in a position that permits expression thereof.
14. A host cell or organism transformed with the expression vector of claim 13 and progeny thereof.
15. E.coli transformed with the expression vector of claim 13 and progeny thereof.
16. A process for making a synthetic mutein comprising culturing the host or progeny of claim 14 and harvesting the synthetic mutein from the culture.
17. A method for making a gene having a DNA sequence that encodes a synthetic mutein of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is non-essential to said biological activity, said mutein having at least one of said cysteine residues substituted by another amino acid and said mutein exhibiting the biological activity of said native protein comprising:
  - (a) hybridizing single-stranded DNA comprising a strand of a structural gene that encodes said protein with a mutant oligonucleotide primer that is complementary to a region of said strand that includes the



codon for said cysteine residue or the anti-sense triplet paired with said codon, as the case may be, except for a mismatch with said codon or said antisense triplet which mismatch defines a triplet that codes for said other amino acid;

(b) extending the primer with DNA polymerizes to form a mutational heteroduplex; and

(c) replicating said mutational heteroduplex

18. The method for making the gene of claim 17 wherein the synthetic mutein has only one of said cysteine residues.

19. The method for making the gene of claim 17 wherein said cysteines of the synthetic mutein are substituted by serine, threonine, glycine, alanine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan or methionine.

20. The method of claim 17 or 18 wherein the mismatch defines a triplet that codes for serine or threonine.

21. The method of claim 17, 18 or 19 wherein the single-stranded DNA is a single-stranded phage that includes said strand and the mutational heteroduplex of step (b) is converted to closed circular heteroduplex.

22. The method of claim 17, 18 or 19 wherein said replicating is effected by transforming a competent bacterial host with the closed circular heteroduplex and culturing the resulting transformants.

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### *Description*

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#### TECHNICAL FIELD

This invention is in the general area of recombinant DNA technology. More specifically it relates to mutationally altered biologically active proteins that differ from their parent analogs by one or more substitutions/deletions of cysteine residues.

#### BACKGROUND ART

Biologically active proteins that are microbially produced via recombinant DNA (rDNA) technology may contain cysteine residues that are nonessential to their activity but are free to form undesirable intermolecular or intramolecular links. One such protein is microbially produced human beta interferon (IFN-.beta.). In the course of the preparation of IFN-.beta. by rDNA techniques, it has been observed that dimers and oligomers of microbially produced IFN-.beta. are formed in E. coli extracts containing high concentrations of IFN-.beta.. This multimer formation renders purification and separation of IFN-.beta. very laborious and time-consuming and necessitates several additional steps in purification and isolation procedures such as reducing the protein during purification and reoxidizing it to restore it to its original conformation, thereby increasing the possibility of incorrect disulfide bond formation. Furthermore, microbially produced IFN-.beta. has also been found to exhibit consistently low specific activity due perhaps to the formation of multimers or of random intramolecular disulfide bridges. It would be desirable, therefore, to be able to alter microbially produced biologically active proteins such as IFN-.beta. in a manner that does not affect their activity adversely but reduces or eliminates their ability to form intermolecular crosslinks or intramolecular bonds that cause the protein to adopt an

undesirable tertiary structure (e.g., a conformation that reduces the activity of the protein).

The present invention is directed to producing by directed mutagenesis techniques mutationally altered biologically active proteins (such proteins are called "muteins", Glossary of Genetics and Cytogenetics, 4th Ed, p 381, Springer-Verlag (1976)) that retain the activity of their parent analogs but lack the ability to form intermolecular links or undesirable intramolecular disulfide bonds. In this regard Shepard, H. M., et al, Nature (1981) 294:563-565 describe a mutein of IFN-.beta. in which the cysteine at position 141 of its amino acid sequence (there are three cysteines in native human IFN-.beta. at positions 17, 31, and 141, Gene (1980) 10:11-15 and Nature (1980) 285:542-547) is replaced by tyrosine. This mutein was made by bacterial expression of a hybrid gene constructed from a partial IFN-.beta. cDNA clone having a G.fwdarw.A transition at nucleotide 485 of the IFN-.beta. gene. The mutein lacked the biological activity of native IFN-.beta. leading the authors to conclude that the replaced cysteine was essential to activity.

Directed mutagenesis techniques are well known and have been reviewed by Lather, R. F. and Lecoq, J. P. in Genetic Engineering Academic Press (1983) pp 31-50. Oligonucleotide-directed mutagenesis is specifically reviewed by Smith, M. and Gillam, S. in Genetic Engineering: Principles and Methods, Plenum Press (1981) 3:1-32.

#### DISCLOSURE OF THE INVENTION

One aspect of the invention is a synthetic mutein of a biologically active protein which protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutein having at least one of said cysteine residues deleted or replaced by another amino acid.

Another aspect of the invention relates to synthetic structural genes having DNA sequences that have been specifically designed ("designer genes") to encode the above described synthetic muteins. Subaspects of this aspect are expression vectors that include such structural designer genes, host cells or organisms transformed with such vectors, and processes for making the synthetic mutein by culturing such transformants or their progeny and recovering the mutein from the culture. In the case of muteins that have therapeutic utility, therapeutic compositions that contain therapeutically effective amounts of the muteins and therapeutic methods are other aspects of the invention.

Another aspect of the invention is a method of preventing a protein having one or more cysteine residues that is free to form an undesirable disulfide link from forming such a link comprising mutationally altering the protein by deleting the cysteine residue(s) or replacing them with other amino acids.

Still another aspect of the invention is a method for making the above described synthetic structural gene by oligonucleotide-directed mutagenesis comprising the following steps:

(a) hybridizing single-stranded DNA comprising a strand of a structural gene that encodes the parent protein with a mutant oligonucleotide primer that is complementary to a region of the strand that includes the codon for the cysteine to be deleted or replaced or the antisense triplet paired with the codon, as the case may be, except for a mismatch with that codon or antisense triplet, as the case may be, that defines a deletion of the codon or a triplet that encodes said other amino acid;

(b) extending the primer with DNA polymerase to form a mutational heteroduplex; and

(c) replicating the mutational heteroduplex.

The mutant oligonucleotide primers used in this process are another aspect of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram of the amino acid sequence of IFN-.beta..

FIG. 2 is a schematic illustration showing the preparation of a mutant IFN-.beta. gene by oligonucleotide-directed mutagenesis.

FIG. 3 shows a diagram of plasmid p.beta.ltrp including the IFN-.beta. gene.

FIG. 4 is a diagram of the cloning vector M13mp8 phage.

FIG. 5 shows the restriction map of clone M13-.beta.1.

FIG. 6 shows the sequencing gel pattern of the mutant IFN-.beta..sub.ser17 gene showing a single base change in the coding region.

FIG. 7 is a diagram of the expression plasmid pTrp3.

FIG. 8a shows the HinfI restriction pattern of clone pSY2501 and FIG. 8b shows the resulting two 169bp and 28bp fragments thereof.

FIG. 9 is a restriction map of clone pSY2501.

FIG. 10 shows the coding DNA sequence for the mutein IFN-.beta..sub.ser17 with the corresponding amino acid sequence therefor.

FIG. 11 shows the single 18,000 dalton protein band corresponding to IFN-.beta..sub.ser17 in the extracts of clones pSY2501 and p.beta.1trp.

FIG. 12 is a diagram of the plasmid pLW1 which contains the human interleukin-2 (IL-2) gene under the control of the E. coli trp promoter

FIG. 13 is a restriction map of phage clone M13-IL2.

FIG. 14 is a restriction map of the plasmid pLW46.

FIGS. 15a and 15b show, respectively, the nucleotide sequence of the coding strand of the clone pLW46 and the corresponding amino acid sequence of the IL-2 mutein designated IL-2.sub.ser125.

FIG. 16 is a diagram of the plasmid pLW32.

FIG. 17 is a diagram of the plasmid pLW55.

FIG. 18 shows the complete nucleotide sequence of pE4, and the deduced amino acid sequence.

FIG. 19 shows a restriction map of the PE4 insert.

FIGS. 20a and 20b show the complete nucleotide sequence of the insert encoding the mature TNF protein in pAW731, and the deduced amino acid sequence, respectively.

FIG. 21 shows the single 17,000 dalton protein band corresponding to ser 69 TNF in the extracts of clone pAW711 and pAW 731.

## MODES FOR CARRYING OUT THE INVENTION

The present invention provides muteins of biologically active proteins in which cysteine residues that are not essential to biological activity have been deliberately deleted or replaced with other amino acids to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation; mutant genes coding for such muteins; and means for making such muteins.

Proteins that may be mutationally altered according to this invention may be identified from available information regarding the cysteine content of biologically active proteins and the roles played by the cysteine residues with respect to activity and tertiary structure. For proteins for which such information is not available in the literature this information may be determined by systematically altering each of the cysteine residues of the protein by the procedures described herein and testing the biological activity of the resulting muteins and their proclivity to form undesirable intermolecular or intramolecular disulfide bonds. Accordingly, while the invention is specifically described and exemplified below as regards muteins of IFN-.beta., IL-2 and tumor necrosis factor (TNF) it will be appreciated that the following teachings apply to any other biologically active protein that contains a functionally nonessential cysteine residue that makes the protein susceptible to undesirable disulfide bond formation. Examples of proteins other than IFN-.beta., TNF, and IL-2 that are candidates for mutational alteration according to the invention are lymphotoxin, colony stimulating factor-1 and IFN-.alpha.1. Candidate proteins sometimes have an odd number of cysteine residues.

The cysteine residues are either deleted or replaced with amino acids which do not affect the biological activity of the resulting mutein. Appropriate amino acid residues are selected from the group consisting of serine, threonine, glycine, alanine valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan, and methionine. Preferred among this group are the residues of serine, threonine, alanine, and valine. Particularly preferred is replacement by a serine residue.

In the case of IFN-.beta. it has been reported in the literature and that both the glycosylated and unglycosylated IFNs show qualitatively similar specific activities and that, therefore, the glycosyl moieties are not involved in and do not contribute to the biological activity of IFN-.beta.. However, bacterially produced IFN-.beta. which is unglycosylated consistently exhibits quantitatively lower specific activity than native IFN-.beta. which is glycosylated. IFN-.beta. is known to have three cysteine residues at positions 17, 31 and 141. Cysteine 141 has been demonstrated by Shepard, et al, supra, to be essential for biological activity. In IFN-.alpha., which contains four cysteine residues, there are two intramolecular --S--S-- bonds: one between cys 29 and cys 138 and another between cys 1 and cys 98. Based on the homology between IFN-.beta. and IFN-.alpha.s cys 141 of IFN-.beta. could be involved in an intramolecular --S--S-- bond with cys 31, leaving cys 17 free to form intermolecular crosslinks. By either deleting cys 17 or substituting it by a different amino acid, one can determine whether cys 17 is essential to biological activity, and its role in --SS-- bond formation. If cys 17 is not essential for the biological activity of the protein, the resulting cys 17-deleted or cys 17-substituted protein might exhibit specific activity close to that of native IFN-.beta. and would possibly also facilitate isolation and purification of the protein.

By the use of the oligonucleotide-directed mutagenesis procedure with a synthetic oligonucleotide primer that is complementary to the region of the IFN-.beta. gene at the codon for cys 17 but which contains single or multiple base changes in that codon, a designer gene may be produced that results in cys 17 being replaced with any other amino acid of choice. When deletion is desired the oligonucleotide



primer lacks the codon for cys 17. Conversion of cys 17 to neutral amino acids such as glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine is the preferred approach. Serine and threonine are the most preferred replacements because of their chemical analogy to cysteine. When the cysteine is deleted, the mature mutein is one amino acid shorter than the native parent protein or the microbially produced IFN-.beta..

Human IL-2 is reported to have three cysteine residues located at positions 58, 105, and 125 of the protein. As in the case of IFN-.beta., IL-2 is in an aggregated oligomeric form when isolated from bacterial cells and has to be reduced with reducing agents in order to obtain a good yield from bacterial extracts. In addition, the purified reduced IL-2 protein is unstable and readily reoxidized upon storage to an oligomeric inactive form. The presence of three cysteines means that upon reoxidation, the protein may randomly form one of three possible intramolecular disulfide bridges, with only one of those being the correct bridge as found in the native molecule. Since the disulfide structure of the native IL-2 protein is not known, it is possible to use the present invention to create mutations at codons 58, 105 and 125 of the IL-2 gene and identify which cysteine residues are necessary for activity and therefore most likely to be involved in native disulfide bridge formation. In the same vein, the cysteine residue that is not necessary for activity can be modified so as to prevent the formation of incorrect intramolecular disulfide bridges and minimize the chance of intermolecular disulfide bridges by removal or replacement of the free cysteine residue.

Human TNF is a 157 amino acid Protein containing two cysteine residues, one at Position 69 and the other at position 101. TNF was first reported by Carswell et al. Proc Natl Acad Sci (U.S.A.) (1975) 72:3666; and has been shown to be cytotoxic selectively to neoplastic cells. TNF has been purified from cell culture, by Matthews, et al Brit J Cancer (1981) 44:418 (from mononuclear phagocytes derived from BCG-injected rabbits) and by Mannel et al, Infect Immun (1980) 30:523, *ibid* (1981) 33:156 from cultures of macrophage enriched peritoneal exudate cells from BCG-infected mice. The sequence encoding TNF produced by the human promyelocytic leukemia cell line (HL-60, ATCC #CCL240) has been cloned and expressed in E coli. and has been shown to have the sequence set forth in FIG. 18.

As will be shown below, neither of the cysteine residues in the TNF sequence appears to be involved in disulfide linkages, and either may be replaced or deleted according to the method of the invention to obtain a stable and biologically active mutein.

The size of the oligonucleotide primer is determined by the requirement for stable hybridization of the primer to the region of the gene in which the mutation is to be induced, and by the limitations of the currently available methods for synthesizing oligonucleotides. The factors to be considered in designing oligonucleotides for use in oligonucleotide-directed mutagenesis (e.g., overall size, size of portions flanking the mutation site) are described by Smith, M. and Gillam S., *supra*. In general the overall length of the oligonucleotide will be such as to optimize stable, unique hybridization at the mutation site with the 5' and 3' extensions from the mutation site being of sufficient size to avoid editing of the mutation by the exonuclease activity of the DNA polymerase. Oligonucleotides used for mutagenesis in accordance with the present invention usually contain from about 12 to about 24 bases, preferably from about 14 to about 20 bases and still more preferably from about 15 to about 18 bases. They will usually contain at least about three bases 3' of the altered or missing codon.

The method for preparing the modified IFN-.beta. gene broadly involves inducing a site-specific mutagenesis in the IFN-.beta. gene at codon 17 (TGT) using a synthetic nucleotide primer which omits the codon or alters it so that it codes for another amino acid. When threonine replaces the cysteine and the primer is hybridized to the antisense strand of the IFN-.beta. gene, the preferred nucleotide primer is GCAATTTTCACTCAG (underlining denotes the altered codon). When it is desirable to delete cysteine, the preferred primer is AGCAATTTTCAGCAGAAGCTCCTG, which omits the TGT codon for cys.

When cysteine is replaced by serine, a 17-nucleotide primer, GCAATTTTCAGAGTCAG, which includes an AGT codon for serine is the primer of choice. The T->A transition of the first base in the cys 17 codon results in changing cysteine to serine. It must be recognized that when deletions are introduced, the proper reading frame for the DNA sequence must be maintained for expression of the desired protein.

The primer is hybridized to single-stranded phage such as M13, fd, or .phi.X174 into which a strand of the IFN-.beta. gene has been cloned. It will be appreciated that the phage may carry either the sense strand or antisense strand of the gene. When the phage carries the antisense strand the primer is identical to the region of the sense strand that contains the codon to be mutated except for a mismatch with that codon that defines a deletion of the codon or a triplet that codes for another amino acid. When the phage carries the sense strand the primer is complementary to the region of the sense strand that contains the codon to be mutated except for an appropriate mismatch in the triplet that is paired with the codon to be deleted. Conditions that may be used in the hybridization are described by Smith, M. and Gillam, S., supra. The temperature will usually range between about 0.degree. C. and 70.degree. C., more usually about 10.degree. C. to 50.degree. C. After the hybridization, the primer is extended on the phage DNA by reaction with DNA polymerase I, T.sub.4 DNA polymerase, reverse transcriptase or other suitable DNA polymerase. The resulting dsDNA is converted to closed circular dsDNA by treatment with a DNA ligase such as T.sub.4 DNA ligase. DNA molecules containing single-stranded regions may be destroyed by S1 endonuclease treatment.

Oligonucleotide-directed mutagenesis may be similarly employed to make a mutant IL-2 gene that encodes a mutein having IL-2 activity but having cys 125 changed to serine 125. The preferred oligonucleotide primer used in making this mutant IL-2 gene when the phage carries the sense strand of the gene is GATGATGCTCTGAGAAAAGGTAATC. This oligonucleotide has a C.fwdarw.G change at the middle base on the triplet that is paired with codon 125 of the IL-2 gene.

Similarly, oligonucleotide directed mutagenesis is employed to obtain a mutant TNF gene that encodes a mutein having TNF activity, but with cys.sub.69 changed to an alternate or deleted amino acid, and/or the cys.sub.101 residue replaced or deleted. For the exemplified conversion of the cys.sub.69 to ser.sub.69, a preferred oligonucleotide primer is 5'-CATGGGTGCTCGGGCTGCCTT-3' This oligonucleotide has a T.fwdarw.A change in the triplet that is paired with codon 69 of the TNF gene. Similarly, cys.sub.101 may be converted to ser.sub.101 with a primer CAAGAGCCCCTCTCAGAGGGAG which contains a corresponding change at the triplet paired with the codon at 101, using ssM13 phage DNA containing the appropriate strand of the human TNF cDNA sequence.

The resulting mutational heteroduplex is then used to transform a competent host organism or cell. Replication of the heteroduplex by the host provides progeny from both strands. Following replication the mutant gene may be isolated from progeny of the mutant strand, inserted into an appropriate expression vector, and the vector used to transform a suitable host organism or cell. Preferred vectors are plasmids pBR322, pCRI, and variants thereof, synthetic vectors and the like. Suitable host organisms are E. coli, Pseudomonas, Bacillus subtilis, Bacillus thuringiensis, various strains of yeast, Bacillus thermophilus, animal cells such as mice, rat or Chinese hamster ovary (CHO) cells, plant cells, animal and plant hosts and the like. It must be recognized that when a host of choice is transformed with the vector, appropriate promoter-operator sequences are also introduced in order for the mutein to be expressed. Hosts may be prokaryotic or eukaryotic (processes for inserting DNA into eukaryotic cells are described in PCT applications Nos. U.S.81/00239 and U.S.81/00240 published 3 Sept. 1981). E. coli and CHO cells are the preferred hosts. The muteins obtained in accordance with the present invention may be glycosylated or unglycosylated depending on the glycosylation occurring in the native parent protein and the host organism used to produce the mutein. If desired, unglycosylated mutein obtained



when *E. coli* or a *Bacillus* is host organism, may be optionally glycosylated in vitro by chemical, enzymatic and other types of modifications known in the art.

In the preferred embodiment of the subject invention respecting IFN- $\beta$ , the cysteine residue at position 17 in the amino acid sequence of IFN- $\beta$ , as shown in FIG. 1, is changed to serine by a T.fwdarw.A transition of the first base of codon 17 of the sense strand of the DNA sequence which codes for the mature IFN- $\beta$ . The site-specific mutagenesis is induced using a synthetic 17-nucleotide primer GCAATTTTCAGAGTCAG which is identical to a seventeen nucleotide sequence on the sense strand of IFN- $\beta$  in the region of codon 17 except for a single base mismatch at the first base of codon 17. The mismatch is at nucleotide 12 in the primer. It must be recognized that the genetic code is degenerate and that many of the amino acids may be encoded by more than one codon. The base code for serine, for example, is six-way degenerate such that the codons, TCT, TCG, TCC, TCA, AGT, and ACG all code for serine. The AGT codon was chosen for the preferred embodiment for convenience. Similarly, threonine is encoded by any one of the codons ACT, ACA, ACC and ACG. It is intended that when one codon is specified for a particular amino acid, it includes all degenerate codons which encode that amino acid. The 17-mer is hybridized to single-stranded M13 phage DNA which carries the antisense strand of the IFN- $\beta$  gene. The oligonucleotide primer is then extended on the DNA using DNA polymerase I Klenow fragment and the resulting dsDNA is converted to closed circular DNA with T.sub.4 ligase. Replication of the resulting mutational heteroduplex yields clones from the DNA strand containing the mismatch. Mutant clones may be identified and screened by the appearance or disappearance of specific restriction sites, antibiotic resistance or sensitivity, or by other methods known in the art. When cysteine is substituted with serine, the T.fwdarw.A transition, shown in FIG. 2, results in the creation of a new *Hinf*I restriction site in the structural gene. The mutant clone is identified by using the oligonucleotide primer as a probe in a hybridization screening of the mutated phage plaques. The primer will have a single mismatch when hybridized to the parent but will have a perfect match when hybridized to the mutated phage DNA, as indicated in FIG. 2. Hybridization conditions can then be devised where the oligonucleotide primer will preferentially hybridize to the mutated DNA but not to the parent DNA. The newly generated *Hinf*I site also serves as a means of confirming the single base mutation in the IFN- $\beta$  gene.

The M13 phage DNA carrying the mutated gene is isolated and spliced into an appropriate expression vector, such as plasmid pTrp3, and *E. coli* strain MM294 is transformed with the vector. Suitable growth media for culturing the transformants and their progeny are known to those skilled in the art. The expressed mutein of IFN- $\beta$  is isolated, purified and characterized.

The following examples are presented to help in the better understanding of the subject invention and for purposes of illustration only. They are not to be construed as limiting the scope of the invention in any manner. Examples 1-11 describe the preparation of a mutein of IFN- $\beta$ . Examples 12-20 describe the preparation of a mutein of IL-2. Examples 21-27 describe the preparation of a TNF mutein, and its assay.

#### EXAMPLE 1

##### Cloning of the IFN-B Gene Into M13 Vector:

The use of M13 phage vector as a source of single-stranded DNA template has been demonstrated by G. F. Temple et al, *Nature* (1982) 296:537-540. Plasmid p.beta.ltrp (FIG. 3) containing the IFN- $\beta$  gene under control of *E. coli* trp promoter, was digested with the restriction enzymes *Hind*III and *Xho*II. The M13mp8 (J. Messing, "Third Cleveland Symposium on Macromolecules: Recombinant DNA," Ed. A. Walton, Elsevier Press, 143-153 (1981)) replicative form (RF) DNA (FIG. 4) was digested with restriction enzymes *Hind*III and *Bam*HI, and mixed with the p.beta.ltrp DNA which had previously been

digested with HindIII and XhoII. The mixture was then ligated with T.sub.4 DNA ligase and the ligated DNA transformed into competent cells of E. coli strain JM 103 and plated on Xgal indicator plates (J. Messing, et al, Nucleic Acids Res (1981) 9:309-321). Plaques containing recombinant phage (white plaques) were picked, inoculated into a fresh culture of JM 103 and minipreps of RF molecules prepared from the infected cells (H. D. Birnboim and J. Doly, Nucleic Acid Res (1979) 7:1513-1523). The RF molecules were digested with various restriction enzymes to identify the clones containing the IFN-.beta. insert. The restriction map of one such clone (M13-.beta.1) is shown in FIG. 5. Single-stranded (ss) phage DNA was prepared from clone M13-.beta.1 to serve as a template for site-specific mutagenesis using a synthetic oligonucleotide.

## EXAMPLE 2

### Site-Specific Mutagenesis;

Forty picomoles of the synthetic oligonucleotide GCAATTTTCAGAGTCAG (primer) was treated with T.sub.4 kinase in the presence of 0.1 mM adenosine triphosphate (ATP), 50 mM hydroxymethylaminomethane hydrochloride (Tris-HCl) pH 8.0, 10 mM MgCl.sub.2, 5 mM dithiothreitol (DTT) and 9 units of T.sub.4 kinase, in 50 .mu.l at 37.degree. C. for 1 hr. The kinased primer (12 pmole) was hybridized to 5.mu.g of ss M13-.beta.1 DNA in 50.mu.l of a mixture containing 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl.sub.2 and 10 mM .beta.-mercaptoethanol, by heating at 67.degree. C. for 5 min and at 42.degree. C. for 25 min. The annealed mixture was then chilled on ice and then added to 50 .mu.l of a reaction mixture containing 0.5 mM each of deoxynucleoside triphosphate (dNTP), 80 mM Tris-HCl, pH 7.4, 8 mM MgCl.sub.2, 100 mM NaCl, 9 units of DNA polymerase I, Klenow fragment, 0.5 mM ATP and 2 units of T.sub.4 DNA ligase, incubated at 37.degree. C. for 3 hr and at 25.degree. C. for 2 hr. The reaction was then terminated by phenol extraction and ethanol precipitation. The DNA was dissolved in 10 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50% sucrose and 0.05% bromophenylblue and electrophoresed on 0.8% agarose gel in the presence of 2 .mu.g/ml of ethidium bromide. The DNA bands corresponding to the RF forms of M13-.beta.1 were eluted from gel slices by the perchlorate method (R. W. Davis, et al, "Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, N.Y., p. 178-179 (1980)). The eluted DNA was used to transform competent JM 103 cells, grown overnight and ssDNA isolated from the culture supernatant. This ssDNA was used as a template in a second cycle of primer extension, the gel purified RF forms of the DNA were transformed into competent JM 103 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

## EXAMPLE 3

### Site Specific Mutagenesis

The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is GCAATTTTCAGACTCAG to change codon 17 of the IFN-.beta. gene from one that codes for cysteine to one that codes for threonine.

## EXAMPLE 4

### Site Specific Deletion

The experiment of Example 2 above is repeated except that the synthetic oligonucleotide primer used is AGCAATTTTCAGCAGAAGCTCCTG to delete codon 17 of the IFN-.beta. gene.

## EXAMPLE 5

## Screening And Identification of Mutagenized Plaques

Plates containing mutated M13-.beta.1 plaques (Example 1) as well as two plates containing unmutated M13-.beta.1 phage plaques, were chilled to 4.degree. C. and plaques from each plate transferred onto two nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2 N NaOH, 1.5 M NaCl and 0.2% Triton X-100 for 5 min, and neutralized by layering onto filter papers soaked with 0.5 M Tris-HCl, pH 7.5 and 1.5 M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2.times.SSC (standard saline citrate), dried and then baked in a vacuum oven at 80.degree. C. for 2 hr. The duplicate filters were prehybridized at 55.degree. C. for 4 hr with 10 ml per filter of DNA hybridization buffer (5.times.SSC) pH 7.0, 4.times.Denhardt's solution (polyvinylpyrrolidone, ficoll and bovine serum albumin, 1.times.=0.02% of each), 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate buffer pH 7.0 and 100 .mu.g/ml of denatured salmon sperm DNA. .sup.32 p-labeled probe was prepared by kinasing the oligonucleotide primer with .sup.32 p-labeled ATP. The filters were hybridized to 3.5.times.10.sup.5 cpm/ml of .sup.32 p-labeled primer in 5 ml per filter of DNA hybridization buffer at 55.degree. C. for 24 hr. The filters were washed at 55.degree. C. for 30 min each in washing buffers containing 0.1% SDS and decreasing amounts of SSC. The filters were washed initially with buffer containing 2.times.SSC and the control filters containing unmutated M13-.beta.1 plaques were checked for the presence of any radioactivity using a Geiger counter. The concentration of SSC was lowered stepwise and the filters washed until no detectable radioactivity remained on the control filters with the unmutated M13-.beta.1 plaques. The lowest concentration of SSC used was 0.1.times.SSC. The filters were air dried and autoradiographed at -70.degree. C. for 2-3 days. 480 plaques of mutated M13-.beta.1 and 100 unmutated control plaques were screened with the kinased oligonucleotide probe. None of the control plaques hybridized with the probe while 5 mutated M13-.beta.1 plaques hybridized with the probe.

One of the five mutated M13-.beta.1 plaques (M13-SY2501) was picked and inoculated into a culture of JM 103. ssDNA was prepared from the supernatant and double-stranded (ds) DNA was prepared from the cell pellet. The ssDNA was used as a template for the dideoxy-sequencing of the clone using the M13 universal primer. The result of the sequence analysis is shown in FIG. 6, confirming that the TGT cys codon has been converted to an AGT ser codon.

## EXAMPLE 6

### Expression of Mutated IFN-.beta. in E. coli

RF DNA from M13-SY2501 was digested with restriction enzymes HindIII and XhoII and the 520 bp insert fragment purified on a 1% agarose gel. The plasmid pTrp3 containing the E. coli trp promoter (FIG. 7) was digested with the enzymes HindIII and BamHI, mixed with the purified M13-SY2501 DNA fragment, and ligated in the presence of T.sub.4 DNA ligase. The ligated DNA was transformed into E. coli strain MM294. Ampicillin resistant transformants were screened for sensitivity to the drug tetracycline. Plasmid DNA from five ampicillin resistant, tetracycline sensitive clones were digested with HinfI to screen for the presence of the M13-SY2501 insert. FIG. 8a shows the HinfI restriction pattern of one of the clones (pSY2501), comparing it with the HinfI pattern of the original IFN-.beta. clone, p.beta.ltrp. As expected, there is an additional HinfI site in pSY2501, cleaving the 197 bp IFN-.beta. internal fragment to a 169 bp fragment and a 28 bp fragment (FIG. 8b). A restriction map of the clone pSY2501 is shown in FIG. 9. The complete DNA sequence of the mutant IPN-.beta. gene is shown in FIG. 10 together with the predicted amino acid sequence.

The plasmid designated as clone pSY2501 was deposited with the Agricultural Research Culture

Collection (NRRL), Fermentation Laboratory, Northern Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, 1815 North University Street, Peoria, Ill. 60604 on 30 Mar. 1983 and was assigned accession numbers CMCC No. 1533 and NRRL No. B-15356.

Cultures of pSY2501 and p.beta.ltrp, which include progeny thereof, were grown up to an optical density (OD.sub.600) of 1.0. Cell free extracts were prepared and the amount of IFN-.beta. antiviral activity assayed on GM2767 cells in a microtiter assay. Extracts of clone pSY2501 exhibited three to ten times higher activity than p.beta.ltrp (Table I), indicating that clone pSY2501 was either synthesizing more protein exhibiting IFN-.beta. activity or that the protein made had a higher specific activity.

TABLE I \_\_\_\_\_ EXTRACT ANTIVIRAL ACTIVITY (U/ml)  
 \_\_\_\_\_ pSY2501 6 .times. 10.sup.5 p.beta.ltrp 1 .times.  
 10.sup.5 ptrp3 (control) 30 \_\_\_\_\_

In order to determine if clone pSY2501 was synthesizing several times more active protein, the extracts of both clones were electrophoresed on a SDS polyacrylamide gel together with a control extract and the gel stained with coomassie blue to visualize the proteins. As shown in FIG. 11, there was only one protein band corresponding to an apparent 18,000 dalton protein that was present in the extracts of clones pSY2501 and p.beta.ltrp but not in the control extract of ptrp3. This protein, which has a molecular weight of about 20,000 daltons but shows a gel migration pattern of an 18,000 dalton protein was previously shown to be IFN-.beta. by purification of this protein from extracts of p.beta.ltrp. Since there is less of this protein in extracts of pSY2501 than in extracts of p.beta.ltrp, the specific activity of the protein in extracts of clone pSY2501 was higher than that of clone p.beta.ltrp.

#### EXAMPLE 7

The plasmid pSY2501 was transformed into a competent subvariant of E. coli strain MM294, designated MM294-1. A sample of the resulting transformant was deposited in the American Type Culture Collection 12301 Parklawn Drive, Rockville, Md. 20852 U.S.A. on 18 Nov. 1983; under ATCC number 39,517.

#### EXAMPLE 8

##### Production of IFN-.beta..sub.ser17

IFN-.beta..sub.ser17 was recovered from E. coli that had been transformed to produce IFN-.beta..sub.ser17. The E. coli were grown in the following growth medium to an OD of 10-11 at 680 nm (dry wt 8.4 g/l).

	Ingredient Concentration
	NH.sub.4 Cl 20 mM K.sub.2 SO.sub.4 16.1 mM
KH.sub.2 PO.sub.4 7.8 mM Na.sub.2 HPO.sub.4 12.2 mM MgSO.sub.4.7H.sub.2 O 3 mM Na.sub.3 citrite.2H.sub.2 O 1.5 mM MnSO.sub.4.4H.sub.2 O 30 .mu.M ZnSO.sub.4.7H.sub.2 O 30 .mu.M CuSO.sub.4.5H.sub.2 O 3 .mu.M L-tryptophan 70 mg/l FeSO.sub.4.7H.sub.2 O 72 .mu.M thiamine.HCl 20 mg/l glucose 40 g/l _____ pH control with NH.sub.4 OH	

A 9.9 l (9.9 kg) harvest of the transformed E. coli was cooled to 20.degree. C. and concentrated by passing the harvest through a cross-flow filter at an average pressure drop of .about.110 kpa and steady-state filtrate flow rate of 260 ml/min until the filtrate weight was 8.8 kg. The concentrate (approximately one liter) was drained into a vessel and cooled to 15.degree. C. The cells in the concentrate were then



disrupted by passing the concentrate through a Manton-Gaulin homogenizer at 5.degree. C., .about.69,000 kpa. The homogenizer was washed with one liter phosphate buffered saline, pH 7.4 (PBS), and the wash was added to the disruptate to give a final volume of two liters. This volume was continuously centrifuged at 12000.times.g at a 50 ml/min flow rate. The solid was separated from the supernatant and resuspended in four liters PBS containing 2% by wt SDS. This suspension was stirred at room temperature for 15 min after which there was no visible suspended mater-The solution was then extracted with 2-butanol at a 1:1 2-butanol:solution volume ratio. The extraction was carried out in a liquid-liquid phase separator using a flow rate of 200 ml/min. The organic phase was then separated and evaporated to dryness to yield 21.3 g of protein. This was resuspended in distilled water at a 1:10 volume ratio.

The recovered product was assayed for human IFN-.beta. activity using an assay based on protection against viral cytopathic effect (CPE). The assay was made in microtiter plates. Fifty .mu.l of minimum essential medium were charged into each well and 25 .mu.l of the sample was placed in the first well and 1:3 volume dilutions were made serially into the following wells. Virus (vesicular stomatitis), cell (human fibroblast line GM-2767), and reference IFN-.beta. controls were included on each plate. The reference IFN-.beta. used was 100 units per ml. The plates were then irradiated with UV light for 10 min. After irradiation 100 .mu.l of the cell suspension (1.2.times.10.sup.5 cells/ml) was added to each well and the trays were incubated for 18-24 hr. A virus solution at one plaque-forming unit per cell was added to each well except the cell control. The trays were then incubated until the virus control showed 100% CPE. This normally occurred 18-24 hr after adding the virus solution. Assay results were interpreted in relation to the location of the 50% CPE well of the reference IFN-.beta. control. From this point the titer of interferon for all samples on the plate was determined. The specific activity of the recovered product was determined to be 5.times.10.sup.7 U/mg.

#### EXAMPLE 9

##### Acid Precipitation And Chromatographic Purification

The process of Example 8 was repeated except that after extraction and separation of the aqueous and organic phases and mixing of the organic phase with PBS at a volume ratio of 3:1 the pH of the mixture was lowered to about 5 by addition of glacial acetic acid. The resulting precipitate was separated by centrifugation at 10000-17000.times.g for 15 min and the pellet was redissolved in 10% w/v SDS, 10 mM DTT, 50 mM sodium acetate buffer, pH 5.5, and heated to 80.degree. C. for 5 min.

The solution was then applied to a Brownlee RP-300, 10 .mu.M, "Aquapore" column using a Beckman gradient system. Buffer A was 0.1% trifluoroacetic acid (TFA) in H.sub.2 O; buffer B was 0.1% TFA in acetonitrile. Detection was by ultraviolet absorbance at 280 nm. The solvent program was linear gradient of 0% buffer B to 100% buffer B in three hr. Fractions containing highest interferon activities were pooled and the specific activity of the pooled interferon preparation was determined to be 9.0.times.10.sup.7 to 3.8.times.10.sup.8 international units per mg protein, as compared to about 2.times.10.sup.8 U/mg for native IFN-.beta..

#### EXAMPLE 10

##### Biochemical Characterization of IFN-.beta.Ser.sub.17

Amino acid compositions were determined after 24-72 hr timed hydrolysis of 40 .mu.g samples of IFN in 200 .mu.l of 5.7 N HCl, 0.1% phenol, at 108.degree. C. Proline and cysteine were determined in the same fashion after performic acid oxidation; in this case, phenol was omitted from the hydrolysis. Tryptophan was analyzed after 24 hr hydrolysis of 400 .mu.l samples in 5.7 N HCl, 10% mercaptoacetic

acid (no phenol). Analysis was performed on a Beckman 121MB amino acid analyzer using a single column of AA10 resin.

The amino acid composition calculated from representative 24-, 48-, 72-hr acid hydrolyses of purified IFN-.beta. Ser.sub.17 agrees well with that predicted by the DNA sequence of the cloned IFN gene, minus the missing N-terminal methionine.

The amino acid sequence of the first 58 residues from the amino acid terminus of purified IFN was determined on a 0.7 mg sample in a Beckman 890C. sequanator with 0.1M Quadrol buffer. PTH amino acids were determined by reverse-phase HPLC on an Altex ultrasphere ODS column (4.6.times.250 mm) at 45.degree. C. eluted at 1.3 min at 40% buffer B, and 8.4 min from 40-70% buffer B, where buffer A was 0.0115M sodium acetate, 5% tetrahydrofuran (THF), pH 5.11 and buffer B was 10% THF in acetonitrile.

The N-terminal amino acid sequence of IFN-.beta. Ser.sub.17 determined matches the expected sequence predicted from the DNA sequence, except for the absence of N-terminal methionine.

#### EXAMPLE 11

##### Alternative IFN-.beta..sub.ser Production and Purification Process

E.coli transformed with pSY2501 were grown in the following medium:

	Approximate Initial Ingredient Concentration
Na.sub.3 Citrate.2H.sub.2 O	3 mM
KH.sub.2 PO.sub.4	30 mM
(NH.sub.4).sub.2 SO.sub.4	74 mM
MgSO.sub.4.7H.sub.2 O	3 mM
MnSO.sub.4.H.sub.2 O	46 .mu.M
ZnSO.sub.4.7H.sub.2 O	46 .mu.M
CuSO.sub.4.5H.sub.2 O	1-2 .mu.M
L-tryptophan	350 .mu.M
FeSO.sub.4.7H.sub.2 O	74 .mu.M
thiamine.HCl	0.002%
glucose	0.5%

Dow Corning Antifoam polypropylene glycol, 25% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

Temperature was maintained at 37.+-.1.degree. C., pH at 6.5.+-.0.1 with NaOH, and dissolved oxygen at 30% of air saturation. Optical density and residual glucose measurements were taken at 14 hr and at approximately one hr intervals thereafter. Harvest was made when glucose consumption reached 40.+-.6 g/l (OD at 680 nm=10-11).

The harvested material was concentrated approximately 3-fold by circulating it through a microporous cross-flow filter under pressure. The concentrated cells were diafiltered against deionized water until the harvest material was concentrated 4-5 fold. The cells were then disrupted by passing them through a Manton-Gaulion homogenizer at .about.4.1-5.5.times.10.sup.4 kpa. After the initial pass SDS-sodium phosphate buffer was added to a final concentration of 2% SDS, 0.08M sodium phosphate and homogenization was continued for one hr. Solid DTT was then added to a final concentration of 50 mM and the homogenizate was heated to 90.+-.5.degree. C. for 10 min. The resulting cell suspension was extracted with 2-butanol at a 1:1 2-butanol:suspension volume ratio in a static mixer. The mixture was then centrifuged and the 2-butanol rich phase was collected.

The 2-butanol rich phase was mixed with 2.5 volumes of 0.1% SDS in PBS. Solid DTT was added to a final concentration of 2 mM. The pH of the mixture was adjusted to 6.2.+-.0.1 with glacial acetic acid and this mixture was centrifuged. The resulting paste was collected and resuspended in PBS +10% SDS



with pH adjustment to 8.5. $\pm$ .0.1 using 1 N NaOH. Solid DTT was added to a final concentration of 100 mM and the suspension was heated to 90. $\pm$ .5.degree. C. for 10 min. The suspension was then cooled to .about.25.degree. C., the pH was adjusted to 5.5. $\pm$ .0.1 with glacial acetic acid, and the solution was filtered.

The solution was then applied to a Sephacryl S-200 pre column and the fractions containing highest interferon activities were pooled and concentrated by ultrafiltration with a 10 Kdal molecular weight cutoff. The concentrate was oxidized by adding equimolar amounts of protein and iodosobenzoic acid into a reaction vessel containing 2 mM sodium pyrophosphate, 0.1% SDS and 1 mM EDTA. The pH was controlled during oxidation at 9.0. $\pm$ .0.1 with 0.5 N NaoH and adjusted to 5.5. $\pm$ .0.2 when oxidation was complete After oxidation the concentrate was again passed through the ultrafiltration unit with a 10 Kdal molecular weight cutoff.

The concentrate was applied to a main Sephacryl S-200 column and the fractions were analyzed by SDS-PAGE to determine those containing no high molecular weight contaminants. Those fractions were pooled and passed through the ultrafiltration unit. The filtered concentrate was then fractionated on a Sephadex G-75 column. SDS-PAGE analysis of the fractions was made to determine those containing no low or high molecular weight contaminants. Those fractions were pooled for desalting.

A Sephadex G-25 column equilibrated with 1 mM NaOH was loaded with the pooled fractions from the Sephadex G-75 column using distilled water adjusted to pH 10.8-11 with 50% NaOH. The purified product was collected as the void volume peak. This desalted, purified IFN-.beta. mutein may be formulated in known manners for therapeutic administration.

#### Biological Testing of IFN-.beta..sub.ser17

##### Antigenic Comparison

IFN-.beta..sub.ser17 was compared antigenically to IFN-.beta. produced from diploid fibroblasts using virus neutralizing tests A polyvalent antiserum to the diploid fibroblast IFN-.beta. was prepared in rabbits. This antiserum blocked the antiviral activity of both the diploid fibroblast IFN-.beta. and the IFN-.beta..sub.ser17 in the virus neutralization tests, indicating the two proteins are indistinguishable antigenically.

##### Antiviral Activity

The purified IFN-.beta..sub.ser17 was compared in its antiviral activity to naturally produced IFN-.beta.. Inhibition of vesicular stomatitis virus replication in diploid foreskin fibroblast (HS27F) was indistinguishable from that of the natural molecule. Similarly, inhibition of herpes simplex virus type 1 in HS27F fibroblasts by the natural and mutant proteins were comparable.

##### Antiproliferative Activity

The antiproliferation activity of IFN-.beta..sub.ser17 for continuous cell lines was compared with that of naturally produced IFN-.beta.. T24 cells derived from a transitional cell carcinoma were treated with 200 units/ml of the proteins. Cell growth was inhibited significantly ( $p < 0.02$ ) by both proteins

##### Natural Killer (NK) Cell Stimulation

The ability of IFN-.beta..sub.ser17 to stimulate NK cell (spontaneous cell mediated cytotoxicity) activity was tested. Ficoll-hypaque separated peripheral human mononuclear cells (PMC) or NK-enriched

lymphocyte preparations (depleted of monocytes by plastic adherence and of OKT3-positive T cells by treatment with OKT3 antibody plus complement) were incubated overnight in growth medium containing various concentrations of IFN-.beta..sub.ser17. .sup.51 Cr-labeled target cells were incubated with the effector cells (effector cell:target cell ratio=50:1) for 2-4 hours. NK cell cytotoxicity was determined by measuring the amount of label released into the medium. The results of these tests are reported in Table I below.

TABLE I

NK Cell Cytotoxicity by Interferon (specific % .sup.51 Cr release .+-. SEM) Target Effector IFN (units/ml) Cell Cells 0 10 30 100 300 1000

																			T24 PMC					
7.23	+-.	5.1	23.1	+-.	4.4	24.4	+-.	1.1	34.1	+-.	2.5	50.0	+-.	2.0	40.4	+-.	4.4	Chang PMC	4.7	+-.	0.5			
7.2	+-.	0.8	9.5	+-.	1.7	15.9	+-.	1.3	21.9	+-.	1.4	26.9	+-.	1.8	Chang NK	Enr	19.2	+-.	4.6	39.4	+-.	4.1		
ND	54.2	+-.	6.1	ND	41.7	+-.	5.5	K562	NK	Enr	41.0	+-.	4.6	48.4	+-.	3.6	ND	62.2	+-.	3.5	ND	63.2	+-.	3.5

As shown the target cells were killed more effectively by the IFN-.beta..sub.ser17 -treated cells than by the untreated cells.

### Clinical Trials

Phase I clinical trials to verify the safety of IFN-.beta..sub.ser17 in humans have been initiated. These trials involve administering the protein to patients intramuscularly and intravenously at doses ranging between 1.times.10.sup.5 units (1 .mu.g of protein) to 400.times.10.sup.6 units. In initial phase I clinical trials no unexpected adverse effects have occurred.

As indicated above, the, IFN-.beta..sub.ser17 preparation exhibits specific activity levels very close to or better than that of native IFN-.beta.. IFN-.beta..sub.ser17 has no free sulfhydryl groups but indicates one --S--S-- bond between the only remaining cysteines at positions 31 and 141. The protein does not readily form oligomers and appears to be substantially in the monomeric form. The IFN-.beta..sub.ser17 obtained in accordance with this invention may be formulated either as a single product or mixtures of the various forms, into pharmaceutically acceptable preparations in inert, nontoxic, nonallergenic, physiologically compatible carrier media for clinical and therapeutic uses in cancer therapy or in conditions where interferon therapy is indicated and for viral infections such as herpes simplex virus I and II, hepatitis B virus, common cold viruses, and rhinovirus. Such media include but are not limited to distilled water, physiological saline, Ringer's solution, Hank's solution and the like. Other nontoxic stabilizing and solubilizing additives such as dextrose, HSA (human serum albumin) and the like may be optionally included. The therapeutic formulations may be administered orally or parenterally such as intravenous, intramuscular, intraperitoneal and subcutaneous administrations. Preparations of the modified IFN-(of the present invention may also be used for topical applications in appropriate media normally utilized for such purposes The IFN-B mutein may be administered either locally or systemically by itself or in combination or conjunction with other therapeutic agents such as cyclovir for prophylactic or therapeutic purposes The dose of mutein administered to human patients will depend on whether it is administered continuously (including intermittent) or as a bolus. The amounts administered continuously will typically be lower than the amounts administered as a bolus. The amount will usually be in the range of about 1.times.10.sup.5 to 4.times.10.sup.8 units, more usually about 1.times.10.sup.6 to 1.times.10.sup.7 units.

The principal advantages of the above described mutein of IFN-.beta.. lie in the elimination of a free sulfhydryl group at position 17 in IFN-.beta., thereby forcing the protein to form correct disulfide links between cys 31 and cys 141 and to assume the conformation ostensibly required for full biological

activity. The increased specific activity of the IFN-.beta..sub.ser17 enables the use of smaller dosages in therapeutic uses. By deleting the cysteine at position 17 and eliminating the free --SH group, the IFN-.beta..sub.ser17 protein does not form dimers and oligomers so readily as the microbially produced IFN-.beta.. This facilitates purification of the protein and enhances its stability.

#### EXAMPLE 12

The nucleotide sequence for a cDNA clone coding for human IL-2, procedures for preparing IL-2 cDNA libraries, and screening same for IL-2 are described by Taniguchi, T., et al, Nature (1983) Vol 24, p 305 et seq.

cDNA libraries enriched in potential IL-2 cDNA clones were made from an IL-2 enriched mRNA fractions obtained from induced peripheral blood lymphocytes (PBL) and Jurkat cells by conventional procedures. The enrichment of the mRNA for IL-2 message was made by fractionating the mRNA and identifying the fraction having IL-2 mRNA activity by injecting the fractions in *Xenopus laevis* oocytes and assaying the oocyte lysates for IL-2 activity on HT-2 cells (J. Watson, J Exp Med (1979) 150:1570-1519 and S. Gillis et al, J Immun (1978) 120:2027-2032.)

#### EXAMPLE 13

##### Screening and Identification of IL-2 cDNA Clones

The IL-2 cDNA libraries were screened using the colony hybridization procedure. Each microtiter plate was replicated onto duplicate nitrocellulose filter papers (S & S type BA-85) and colonies were allowed to grow at 37.degree. C. for 14-16 hr on L agar containing 50 .mu.g/ml ampicillin. The colonies were lysed and DNA fixed to the filter by sequential treatment for 5 min with 500 mM NaOH, 1.5 M NaCl, washed twice for 5 min each time with 5.times. standard saline citrate (SSC). Filters were air dried and baked at 80.degree. C. for 2 hr. The duplicate filters were pre-hybridized at 42.degree. C. for 6-8 hr with 10 ml per filter of DNA hybridization buffer (50% formamide, 5.times.SSC, pH 7.0, 5.times.Denhardt's solution (polyvinylpyrrolidone, plus ficoll and bovine serum albumin; 1.times.=0.2% of each), 50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, 20 .mu.g/ml Poly U, and 50 .mu.g/ml denatured salmon sperm DNA.

A 32P-labeled 20-mer oligonucleotide probe was prepared based on the IL-2 gene sequence reported by Taniguchi, T., et al, supra. The nucleotide sequence of the probe was GTGGCCTTCTTGGGCATGTA.

The samples were hybridized at 42.degree. C. for 24-36 hr with 5 ml/filter of DNA hybridization buffer containing the .sup.32 P cDNA probe. The filters were washed two times for 30 min each time at 50.degree. C. with 2.times.SSC, 0.1% SDS, then washed twice with 1.times.SSC and 0.1% SDS at 50.degree. C. for 90 min, air dried, and autoradiographed at -70.degree. C. for 2 to 3 days. Positive clones were identified and rescreened with the probe. Full length clones were identified and confirmed by restriction enzyme mapping and comparison with the sequence of the IL-2 cDNA clone reported by Taniguchi, T., et al, supra.

#### EXAMPLE 14

##### Cloning of IL-2 Gene into M13 Vector

The IL-2 gene was cloned into M13mp9 as described in Example 1 using the plasmid pLW1 (FIG. 12) containing the IL-2 gene under the control of the E. coli trp promoter. A sample of pLW1 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A., on 4

Aug. 1983 and has been assigned ATCC No. 39,405. The restriction map of one clone (designated M13-IL2) containing the IL-2 insert is shown in FIG. 13. Single-stranded phage DNA was prepared from clone M13-IL2 to serve as a template for oligonucleotide-directed mutagenesis.

#### EXAMPLE 15

##### Oligonucleotide-directed Mutagenesis

As indicated previously, IL-2 contains cysteine residues at amino acid positions 58, 105 and 125. Based on the nucleotide sequences of the portions of the IL-2 gene that contain the codons for these three cysteine residues three oligonucleotide primers were designed and synthesized for mutating the codons for these residues to codons for serine. These oligonucleotides have the following sequences.

CTTCTAGAGACTGCAGATGTTTC (DM27) to change cys 58,

CATCAGCATACTCAGACATGAATG (DM28) to change cys 105 and

GATGATGCTCTGAGAAAAGGTAATC (DM29) to change cys 125.

Forty picomoles of each oligonucleotide were kinased separately in the presence of 0.1 mM ATP, 50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mM DTT and 9 units of T<sub>4</sub> kinase in 50  $\mu$ l at 37.degree. C. for 1 hr. Each of the kinased primers (10 pmoles) was hybridized to 2.6  $\mu$ g of ss M13-IL2 DNA in 15  $\mu$ l of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM MgCl<sub>2</sub> and 20 mM  $\beta$ -mercaptoethanol, by heating at 67.degree. C. for 5 min and 42.degree. C. for 25 min. The annealed mixtures were chilled on ice and then adjusted to a final volume of 25  $\mu$ l of a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM MgCl<sub>2</sub>, 83 mM NaCl, 17 mM  $\beta$ -mercaptoethanol, 5 units of DNA polymerase I Klenow fragment, 0.5 mM ATP and 2 units of T<sub>4</sub> DNA ligase, incubated at 37.degree. C. for 5 hr. The reactions were terminated by heating to 80.degree. C. and the reaction mixtures used to transform competent JM103 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

#### EXAMPLE 14

##### Screening and Identification of Mutagenized Phage Plaques

Plates containing mutagenized M13-IL2 plaques as well as 2 plates containing unmutagenized M13-IL2 phage plaques, were chilled to 4.degree. C. and phage plaques from each plate were transferred onto two nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2 N NaOH, 1.5 M NaCl and 0.2% Triton for 5 min, and neutralized by layering onto filter papers soaked with 0.5 M Tris-HCl, pH 7.5, and 1.5 M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2.times.SSC, dried and then baked in a vacuum oven at 80.degree. C. for 2 hr. The duplicate filters were pre-hybridized at 42.degree. C. for 4 hr with 10 ml per filter of DNA hybridization buffer (5.times.SSC, pH 7.0, 4 .times.Denhardt's solution (polyvinylpyrrolidone, ficoll and bovin serum albumin, 1.times.=0.02% of each), 0.1% SDS, 50 mM sodium phosphate buffer, pH 7.0 and 100  $\mu$ g/ml of denatured salmon sperm DNA. <sup>32</sup>P-labelled probes were prepared by kinasing the oligonucleotide primers with labelled ATP. The filters were hybridized to 0.1.times.10<sup>5</sup> cpm/ml of <sup>32</sup>P-labelled primers in 5 ml per filter of DNA hybridization buffer at 42.degree. C. for 8 hr. The filters were washed twice at 50.degree. C. for 30 min each in washing buffers containing 0.1% SDS and 2.times.SSC, and twice at 50.degree. C. for 30 min each with 0.1% SDS and 0.2.times.SSC. The filters were air dried and autoradiographed at -70.degree. C. for 2-3 days.



Since the oligonucleotide primers DM28 and DM29 were designed to create a new DdeI restriction site in the mutagenized clones (FIG. 14), RF-DNA from a number of the clones which hybridized with each of these kinased primers were digested with the restriction enzyme DdeI. One of the mutagenized M13-IL2 plaques which hybridized with the primer DM28 and has a new DdeI restriction site (M13-LW44) was picked and inoculated into a culture of JM103, ssDNA was prepared from the culture supernatant and dsRF-DNA was prepared from the cell pellet. Similarly, a plaque which hybridized with primer DM29 was picked (M13-LW46) and ssDNA and RF-DNA prepared from it. The oligonucleotide primer DM27 was designed to create a new PstI restriction site instead of a DdeI site. Therefore, the plaques that hybridized to this primer were screened for the presence of a new PstI site. One such phage plaque was identified (M13-LW42) and ssDNA and RF-DNA prepared from it. The DNA from all three of these clones were sequenced to confirm that the target TGT codons for cysteine had been converted to a TCT codon for serine.

#### EXAMPLE 17

##### Recloning of the Mutagenized IL-2 Gene for Expression in E. coli

RF-DNA from M13-LW42, M13-LW44 and M13-LW46 were each digested with restriction enzymes HindIII and BanII and the insert fragments purified from a 1% agarose gel. Similarly, the plasmid pTrp3 (FIG. 7) was digested with HindIII and BanII, the large plasmid fragment containing the trp promoter was purified on an agarose gel and then ligated with each of the insert fragments isolated from M13-LW42, M13-LW44 and M13-LW46. The ligated plasmids were transformed into competent E. coli K12 strain MM294. The plasmid DNAs from these transformants were analyzed by restriction enzyme mapping to confirm the presence of the plasmids pLW42, pLW44 and pLW46. FIG. 14 is a restriction map of pLW46. When each of these individual clones were grown in the absence of tryptophane to induce the trp promoter and cell free extracts analyzed on SDS-polyacrylamide gels, all three clones, pLW42, pLW44 and pLW46, were shown to synthesize a 14.5 kd protein similar to that found in the positive control, pLW21, which has been demonstrated to synthesize a 14.4 kd IL-2 protein. When these same extracts were subjected to assay for IL-2 activity on mouse HT-2 cells, only clones pLW21 (positive control) and pLW46 had significant amounts of IL-2 activity (Table II below), indicating that cys 58 and cys 105 are necessary for biological activity and changing them to serines (pLW42 and pLW44 respectively) resulted in the loss of biological activity. Cys 125 on the other hand must not be necessary for biological activity because changing it to ser 125 (pLW46) did not affect the biological activity.

TABLE II		Clones IL-2 Activity (.mu./ml)	
		pIL2-7 (negative control)	1 pLW21 (positive control)
113,000	pLW42 660	pLW44 1,990	pLW46 123,000

FIG. 15a shows the nucleotide sequence of the coding strand of clone pLW46. As compared to the coding strand of the native human IL-2 gene clone pLW46 has a single base change of G.fwdarw.C at nucleotide 374. FIG. 15b shows the corresponding amino acid sequence of the IL-2 mutein encoded by pLW46. This mutein is designated IL-2.sub.ser125 As compared to native IL-2 the mutein has a serine instead of a cysteine at position 125, has an initial N-terminal methionine (which is processed off), and lacks the initial N-terminal alanine of the native molecule.

A sample of E. coli K12 strain MM294 transformed with pLW46 was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. on 26 Sept. 1983 and has been assigned ATCC No. 39,452.

Examples 18 and 19 describe the preparation of an alternative and preferred vector for expressing IL-2.sub.ser125.

#### EXAMPLE 18

##### Construction of Ala-IL-2 Expression Vector pLW32

A codon (GCG) for alanine was inserted immediately after the initiation codon of the IL-2 gene of pLW1 by oligonucleotide-directed mutagenesis as follows. The oligonucleotide primer, 5'-GAAGTAGGCGCCATAAG-3', was kinased, hybridized to ssM13-IL2 DNA, and extended using the general procedure of Example 15 to form a mutational heteroduplex. In addition to the insertion of the GCG codon, the mutagenesis generated a new NarI restriction site in the gene. The heteroduplex was converted to closed circular heteroduplex and the circular heteroduplexes were used to transform competent JM103 cells and plated onto agar plates and incubated as in Example 15. The plates were screened to identify mutagenized M13-IL2 by the procedure of Example 16. One mutagenized phage, identified as M13-LW32, was selected for use in additional cloning and RF-DNA was prepared from it. FIG. 16 is a diagram of plasmid pLW32.

#### EXAMPLE 19

##### Construction of Ala-IL-2.sub.ser125 Expressing Clone pLW55

RF-DNA from M13-LW46 (Examples 16 and 17) was digested with XbaI and PstI and the 530 bp fragment containing the carboxy terminal coding region of the IL-2.sub.ser125 gene was purified from an agarose gel. Similarly, pLW32 was digested with XbaI and PstI and the large fragment consisting of the plasmid vector and the ala-IL-2 N-terminal coding sequence was purified. The two purified DNA fragments were pooled and ligated using T.sub.4 DNA ligase. The ligated DNA was transformed into competent E. coli K-12 strain MM294. Tetracycline resistant transformants were analyzed by restriction enzyme mapping for the presence of a plasmid containing an ala-IL-2.sub.ser125 gene, identified as pLW55, which has a new DdeI site not found in pLW32. FIG. 17 is a diagram of pLW55. Cell free extracts of bacterial culture containing pLW55 were found to contain over 10.sup.5 units of IL-2 activity per ml by the HT-2 cell assay, J. Watson, supra, and S. Gillis, supra. Ala-IL-2.sub.ser125 protein is identical to the IL-2.sub.ser125 molecule shown in FIG. 15(b) except that the former includes the initial N-terminal alanine of the native molecule.

A sample of E. coli K-12 strain MM294 transformed with pLW55 was deposited in the American Type Culture Collection on 18 Nov. 1983 and has been assigned ATCC No. 39,516.

#### EXAMPLE 20

##### Ala-IL-2.sub.ser125 Production and Purification

E. coli transformed with pLW55 were grown in a fermenter containing the following medium:

\_\_\_\_\_(NH.sub.4).sub.2 SO.sub.4 150 mM KH.sub.2 PO.sub.4  
21.6 mM Na.sub.3 Citrate 1.5 mM ZnSO.sub.4.7H.sub.2 O 30 mM MnSO.sub.4.H.sub.2 O 30 mM  
CuSO.sub.4.5H.sub.2 O 1 mM \_\_\_\_\_

pH adjusted to 6.50 with 2.5 N NaOH autoclaved

\_\_\_\_\_  
Sterile Additions (post autoclave)



MgSO<sub>4</sub>·7H<sub>2</sub>O 3 mM FeSO<sub>4</sub> 100 .mu.M  
 L-tryptophan 14 mg/l Thiamine-HCl 20 mg/l Glucose 5 g/l Tetracycline 5 mg/l Ethanol 2% Casamino  
 acids 2%

Dow Corning Antifoam polypropylene glycol, 20% solution, glucose, 50% solution, and KOH, 5N, were added on demand.

The pH of the fermenter was maintained at 6.8 with 5 N KOH. Residual glucose was maintained between 5-10 g/l, dissolved oxygen at 40%, and temperature at 37 .+-.1.degree. C. The casamino acids (20% stock solution) to a concentration of 2% were added when the OD<sub>680</sub> was about 10. Harvest was made three hr after the OD reached about 20.

The harvested material was concentrated and homogenized as in Example 11. Following DTT-heat treatment, the material was centrifuged and the resulting paste was extracted with urea to a final concentration of 4M. The suspension was centrifuged and SDS was added to the solid phase to a concentration of 5%.

The solution was applied to a Sephacryl S-200 column and fractions containing IL-2 (by SDSPA) were pooled. The pooled fractions were applied to a Whatman M-40 column packed with 18 micron Vydac C<sub>18</sub> 300 .ANG. pore size bonded phase silica gel equilibrated in 0.1% TFA. The IL-2 mutein was eluted with a gradient of 40% to 60% 2-propanol, containing 0.1% TFA, in 160 min. Fractions containing highest IL-2 activities were pooled and found to have specific activities comparable to native IL-2.

Muteins of IL-2 in which the cysteine at position 125 has been deleted or replaced with another amino acid, such as the mutein IL-2<sub>ser125</sub> retain IL-2 activity. They may, therefore, be formulated and used in the same manner as native IL-2. Accordingly, such IL-2 muteins are useful for the diagnosis and treatment (local or systemic) of bacterial, viral, parasitic, protozoan and fungal infections; for augmenting cell-mediated cytotoxicity; for stimulating lymphokine activated killer cell activity; for mediating recovery of immune function of lymphocytes; for augmenting alloantigen responsiveness; for facilitating recovery of immune function in acquired immune deficient states; for reconstitution of normal immunofunction in aged humans and animals; in the development of diagnostic assays such as those employing enzyme amplification, radiolabelling, radioimaging, and other methods known in the art for monitoring IL-2 levels in the diseased state; for the promotion of T cell growth in vitro for therapeutic and diagnostic purposes for blocking receptor sites for lymphokines; and in various other therapeutic, diagnostic and research applications. The various therapeutic and diagnostic applications of human IL-2 have been investigated and reported in S. A. Rosenberg, E. A. Grimm, et al, A. Mazumder, et al, and E. A. Grimm and S. A. Rosenberg. IL-2 muteins may be used by themselves or in combination with other immunologically relevant B or T cells or other therapeutic agents. Examples of relevant cells are B or T cells, natural killer cells, and the like and exemplary therapeutic reagents which may be used in combination with the polypeptides of this invention are the various interferons, especially gamma interferon, B cell growth factor, IL-1 and the like. For therapeutic or diagnostic applications, they may be formulated in nontoxic, nonallergenic, physiologically compatible carrier media such as distilled water, Ringer's solution, Hank's solution, physiological saline and the like. Administrations of the IL-2 muteins to humans or animals may be oral or intraperitoneal or intramuscular or subcutaneous as deemed appropriate by the physician. The amount of IL-2 mutein administered will usually range between about 1.times.10<sup>4</sup> and 2.times.10<sup>8</sup> units.

#### EXAMPLE 21

##### Preparation and Purification of Human TNF

## 1. Induction of TNF

High density (1.2 x 10<sup>6</sup> cells/ml) stationary HL-60 cells were centrifuged washed with RPMI 1640 medium in the absence of serum. and then resuspended at a density of 1 x 10<sup>7</sup> cells/ml. The cells were then treated with 100 ng/ml of a phorbol ester, 12-O-tridecanoylphorbol-13-acetate (TPA) for 30 min at 37 degree C. in a suspension culture with constant agitation. The cultures were centrifuged the supernatant was decanted the cells were resuspended at 1 x 10<sup>7</sup> cells/ml in RPMI containing 10 µg/ml bacterial lipopolysaccharide (LPS) and 10 µM Ca ionophore (A23817) for 4 hr at 37 degree C. with constant agitation. The cells were spun down at 1200 rPm for 10 min. and the supernatants recentrifuged at 8000 rPm for 20 min. The resulting supernatant was used in the Purification scheme below to obtain native TNF.

## 2. Purification of TNF

About 4-8 liters of the supernatant prepared from induced HL-60 in the previous paragraph were concentrated via Amicon hollow fiber (1 square foot cartridge/10,000 MW cutoff) to approximately 300 ml. The concentrated culture fluid was centrifuged to remove cell debris, and supernatant adjusted with 30 mM ammonium bicarbonate buffer (pH 8.2) to a conductance of 6.2 mS. The solution was further concentrated by ultrafiltration using a PM10 (Amicon) membrane, and the concentrated fluid clarified by centrifugation (20,000.g for 10 min).

The supernatant was then applied to a DEAE ion exchange column equilibrated in 30 mM ammonium bicarbonate/1 mM NaCl pH 8.2, and the column washed with the same buffer. Fractions were collected and Protein monitored at 280 nm. These unbound fractions were assayed using the L-929 cytotoxicity assay and those having TNF activity pooled and again concentrated by ultrafiltration.

The concentrate was applied to Sephadex G75 Superfine (Pharmacia) equilibrated in 30 mM ammonium bicarbonate buffer (PH 7.4). Unbound fractions obtained by washing With the same buffer were monitored at 280 nm and assayed for TNF. Fractions containing Peak TNF bioactivity Were lyophilized

The lyophilized protein was resuspended in Laemmli SDS sample buffer and electrophoresed on SDS-Polyacrylamide gel. The gel was sliced into 2 mm sections, and the protein from each section was eluted by immersion in 1 ml of 30 mM ammonium bicarbonate buffer (PH 7.4) and overnight shaking at room temperature.

The sections containing the TNF bioactivity were applied onto a Vydac C-4 reverse phase HPLC column equilibrated in 0.1% trifluoroacetic acid (TFA). and the activity eluted using a linear gradient 0%-60% acetonitrile in 0.1% TFA. Protein was monitored at 280 nm and 214 nm. and the fractions bioassayed after lyophilization and suspended in 30 mM ammonium bicarbonate buffer PH 7.4. Fractions containing TNF activity were again lyophilized.

The resulting Protein was of sufficient Purity to be in sequence analysis. The sequence was determined using a gas Phase sequenator (Applied Biosystems Inc.). The sequence obtained from the first 22 amino acids is shown below. ##STR1##

In addition, the Purified Protein (from the G-75 gel) was tested with a modification of the L-929 cytotoxicity assay using alternate human tumor and normal cell lines as substrate. The G-75 fractions which were cytotoxic in this assay against L-929 cells were also cytotoxic against Hs939T (a melanoma line) BT-20 (breast carcinoma). A427 (lung carcinoma) HT-1080 (colon carcinoma) and HT-29 (colon carcinoma). These fractions were not cytotoxic against Hs939sk (skin fibroblasts), HeLa cells (cervical

carcinoma) Hs27F (foreskin fibroblasts) or COS7 (SV40-transformed monkey cells).

## EXAMPLE 22

### Preparation of the Coding Sequence

An intronless DNA sequence encoding human TNF was prepared by the procedure herein described. A human promyelocytic leukemia cell line which produces large amounts of TNF when induced, the HL-60 line, obtainable from ATCC, accession no. CCL 240, was used as the source of mRNA to obtain a cDNA library. Using oligomeric probes constructed on the basis of the protein sequence determined from TNF purified from these cells, this cDNA library was probed to retrieve the entire coding sequence for the protein.

#### 1. Preparation of Enriched mRNA

Total messenger RNA was extracted and purified from HL-60 cells as follows: HL-60 cells were induced for TNF production as set forth in D.I.a. and the 4-hr cell suspension harvested by centrifugation. Total cytoplasmic ribonucleic acid (RNA) was isolated as follows; all steps are at 4.degree. C. Cells are washed twice in pBS (phosphate buffered saline) and resuspended in IHB (140 mM NaCl, 10 mM Tris, 1.5 mM MgCl.sub.2, pH 8) containing 10 mM vanadyl adenosine complex (Berger, S. L., et al, Biochem (1979) 18:5143).

A non-ionic detergent of the ethylene oxide polymer type (NP-40) was added to 0.3% to lyse the cellular but not nuclear membranes. Nuclei were removed by centrifugation at 1,000.times.g for 10 min. The post-nuclear supernatant was added to an equal volume of TE (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5) saturated phenol/chloroform (1:1) containing 0.5% sodium dodecyl sulfate (SDS) and 10 mM EDTA. The supernatant was re-extracted 4 times and phase separated by centrifugation at 2,000.times.g for 10 min. The RNA was precipitated by adjusting the sample to 0.25 M NaCl, adding 2 volumes of 100% ethanol and storing at -20.degree. C. The RNA was pelleted at 5,000.times.g for 30 min. washed with 70% and 100% ethanol and dried. Polyadenylated (Poly A+) messenger RNA (mRNA) was obtained from the total cytoplasmic RNA by chromatography on oligo dT cellulose (Aviv, J., et al, Proc Natl Acad Sci (1972) 69:1408-1412): The RNA was dissolved in ETS (10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.5) at a concentration of 2 mg/ml. This solution was heated to 65.degree. C. for 5 min. then quickly chilled to 4.degree. C. After bringing the RNA solution to room temperature, it was adjusted to 0.4M NaCl and slowly passed through an oligo dT cellulose column previously equilibrated with binding buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5). The flow-through was passed over the column twice more, and the column washed with 10 volumes of binding buffer. Poly A.sup.+ mRNA was eluted with aliquots of ETS, extracted once with TE-saturated phenol chloroform and precipitated by the addition of NaCl to 0.2M and 2 volumes of 100% ethanol. The RNA was reprecipitated twice, washed once in 70% and then in 100% ethanol prior to drying.

The poly A.sup.+ mRNA was fractionated on a sucrose gradient in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM NaCl and 0.1% SDS. After centrifugation in a Beckman SW40 rotor at 38,000 rpm for 17 hr, mRNA fractions were recovered from the gradient by ethanol precipitation. The fractions containing TNF mRNA were identified by injecting the mRNA into oocytes and assaying the oocyte extracts for cytotoxic activity. Fractions containing peak activity were pooled for use in cDNA library construction.

#### 2. Construction of a cDNA Library

cDNA was made from the enriched 16S mRNA fraction using oligo dT priming of the poly A tails and

AMV reverse transcriptase employing the method of Okayama, H., et al, Mol Cell Biol (1983) 3:280, incorporated herein by reference. This method results in a higher proportion of full length clones and effectively uses as host vector portions of two vectors therein described, and readily obtainable from the authors, pcDV1 and pL1. The resulting vectors contain the insert between vector fragments containing proximal BamHI and XhoI restriction sites; the vector contains the pBR322 origin of replication, and Amp resistance gene.

Other methods of preparing cDNA libraries are of course, well known in the art. One, now classical method uses oligo dT primer, reverse transcriptase, tailing of the double stranded cDNA with poly dG. and annealing into a suitable vector, such as pBR322 or a derivative thereof, which has been cleaved at the desired restriction site and tailed with poly dC. A detailed description of this alternate method is found, for example, in U.S. Ser. No. 564,224, filed Dec. 20, 1983 and assigned to the same assignee incorporated herein by reference.

In the method used here, the enriched mRNA (5 .mu.g) was denatured by treatment with 10 mM methyl mercury at 22.degree. C. for 5 min and detoxified by the addition of 100 mM 2-mercaptoethanol (payvar. F., et al, J Biol Chem (1979) 254:7636-7642). Plasmid pcDVI was cleaved with KpnI, tailed with dTTP, and annealed to the denatured mRNA. This oligo dT primed mRNA was treated with reverse transcriptase and the newly synthesized DNA strand tailed with dCTP. Finally, the unwanted portion of the pcDV1 vector was removed by cleavage with HindIII. Separately, pL1 was cleaved with PstI, tailed with dGTP, cleaved with HindIII, and then mixed with the poly T tailed mRNA/cDNA complex extended by the pcDV1 vector fragment ligated with E. coli ligase and the mixture treated with DNA polymerase I (Klenow) E. coli ligase and RNase H. The resulting vectors are transformed into E. coli K12 MM294 to Amp.RTM..

### 3. Selection of probe

Oligomers complementary to the coding sequence for amino acids 8-12 of the purified TNF sequence were prepared. Because of codon redundancy, a total of sixty-four 14-mers are candidates for complementation to the messenger encoding this portion. All sixty-four 14-mers were prepared, and divided into four pools of sixteen. Each pool was mixed with the sucrose gradient size-fractionated enriched mRNA preparation prepared as above, and the mixture injected into the oocyte translation system. Controls were run using untreated messenger RNA. The proteins produced in the oocyte systems were subjected to L-929 cytotoxicity assay (.sup.35 S release) and the proteins derived from oocytes injected with control and with a mixture of mRNA with three of the oligomer pools showed activity. In this "hybrid arrest" assay, only the oocyte injected with messenger which had been treated with the pool having the sequence ##STR2## was inactive. The specificity of this oligomer pool was further determined using "dot blot" hybridization with enriched mRNA prepared as above from both induced and uninduced HL-60 cells, as well as the corresponding mRNA fraction obtained from cells known to be producers of lymphotoxin. This pool hybridized well to the induced mRNA, but failed to hybridize with the corresponding fractions from the uninduced or lymphotoxin producing cells. However Northern blots using the kinased pool as probe demonstrated that it contained sequences which cross hybridize with the 18S (ribosomal) RNA fraction and to pBR322 DNA.

The successful pool was therefore further fractionated by synthesizing its members as eight pairs of 14-mers, each of which was used in the "hybrid arrest" assay performed as set forth above. Only the pair with the sequence ##STR3## was successful in inhibiting the synthesis of TNF in the oocytes. Dot blot experiments using the fractionated induced HL-60 mRNA fraction, induced total HL-60 poly A.sup.+ RNA, uninduced HL-60 poly A.sup.+ RNA, and pBR322 DNA confirmed the specificity of the foregoing 14-mer pair and the inability of the remaining pairs to hybridize to the desired messenger.



#### 4. Recovery of the Coding Sequence

The cDNA library was probed with the 14-mer pair identified above. Twenty-eight colonies which hybridized with probe were picked cultured, and the plasmid DNA isolated. Plasmids containing inserts of sufficient length to encode the entire sequence were selected and several were assayed for the correct sequence using hybrid translation in combination with the .sup.35 S release version of the cytotoxic assay, as described below. Hybrid translation assays use the test sequence to retrieve the correct mRNA from unfractionated preparations as verified by assaying the protein produced by the oocyte translation system injected with the retrieved messenger.

The plasmid cDNA to be tested is bound to filters, and the filters treated with poly A.sup.+ RNA isolated from induced HL-60 cells. The filters are then eluted and the eluates injected into the oocyte translation system. The oocytes are extracted for protein, which is then tested in the .sup.35 S version of the L-929 cytotoxic assay. The results for several hybridizing clones, designated E2-E4 E6 and E8 are shown below:

	Sample % Release of .sup.35 S												
	E1	7	E2	23	E3	32	E4	33	E6	26	E8	11	pBR322 9 A+ 34
B+ 24													

(A+ and B+ are controls using enriched mRNA as obtained by sucrose gradient; E1 and pBR322 are negative controls.)

Restriction analysis and partial sequencing of the inserts indicated that two candidate plasmids, pE4 and pB11 were likely to have the complete TNF encoding sequence. The results of this analysis for pE4 are shown in FIG. 19. PE4 was deposited at ATCC on Oct. 15, 1984 and has accession No. 39,894.

pE4 was sequenced and the correct reading frame for TNF identified by matching the amino acid sequence deduced from translation of all three possible reading frames with the known N-terminal sequence of the native mature TNF as determined by N-terminal sequencing of the purified protein (see FIG. 18). The amino acids in the mature protein are numbered starting with the valine at position 1. As noted above, homology was not complete. However, the high degree of homology indicated that the correct cDNA had been chosen. Verification of the experimentally determined restriction cleavage sites shown in FIG. 19 is also provided. The HindIII site upstream of the 3' PstI site in the 1.1 kb PstI fragment is downstream of the stop codon, thus permitting excision of the coding sequence as a HindIII cassette, after modification of the upstream region as described below.

#### 5. Characteristics of Human TNF as Determined from the DNA Sequence

As deduced from the cDNA sequence set forth in FIG. 18, the mature TNF protein contains 1-57 amino acid residues and has a molecular weight, without glycosylation, of approximately 17,354. The leader sequence apparently contains roughly 76 amino acids, beginning with the first available Met start codon. There are 2 cysteine residues, at positions 69 and 101.

#### EXAMPLE 23

##### Modification of the N-terminal Codons in pE4

It was convenient, in effecting the expression of the mature protein, to introduce an ATG start codon immediately preceding the GTC sequence encoding N-terminal valine of the mature protein (designated 1 in FIG. 18). as well as to provide a HindIII site immediately upstream of the ATG for ligation into

suitable host expression vectors. This was accomplished by site-directed mutagenesis in a manner analogous to that described in Examples 1, 2 and 5.

The DNA fragment containing the upstream portion of the coding sequence was excised from pE4 by digestion with PstI, isolated by agarose gel electrophoresis, recovered by electroelution, and ligated into the PstI site of bacteriophage M13mp18.

The ligated phage were transduced into frozen competent E.coli K12 strain DG98 (ATCC #39768) and cultured by plating on media containing 5.times.10<sup>sup</sup>.-4 M isopropyl thiogalactoside (IPTG) obtained from Sigma Chem. (St Louis, Mo.) and 40 .mu.g/ml X-gal. Non .alpha.-complementing white plaques were picked onto fresh media. Mini-cultures were screened for recombinant single strand phage DNA containing inserts of the expected (1.1 kb) size. The structure of the desired recombinant phage, designated clone 4.1, was confirmed using restriction analysis.

A chemically synthesized. Purified. 33-mer oligodeoxyribonucleotide having the sequence:

was used to introduce a HindIII restriction enzyme site and an ATG-initiation codon before the GTC codon coding for the first amino acid (valine) of the mature TNF protein.

Ten picomoles of the oligonucleotide were hybridized to 2.6 .mu.g of ss clone 4.1 DNA in 15 .mu.l of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM MgCl<sub>sub</sub>.2 and 20 mM .beta.-mercaptoethanol, by heating at 67.degree. C. for 5 min and 42.degree. C. for 25 min. The annealed mixtures were chilled on ice and then adjusted to a final volume of 25 .mu.l of a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM MgCl<sub>sub</sub>.2, 83 mM NaCl, 17 mM .beta.-mercaptoethanol, 5 units of DNA polymerase I Klenow fragment, incubated at 37.degree. C. for 1 hr. The reactions were terminated by heating to 80.degree. C. and the reaction mixtures used to transform competent DG98 cells. Plated onto agar plates and incubated overnight to obtain phage plaques.

Plates containing mutagenized clone 4.1 plaques as well as 2 plates containing unmutagenized clone 4.1 phage plaques. Were chilled to 4.degree. C. and phage plaques from each plate were transferred onto 2 nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2N NaOH, 1.5M NaCl and 0.2% Triton X-100 for 5 min, and neutralized by layering onto filter papers soaked with 0.5M Tris-HCl, pH 7.5, and 1.5M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2.times.SSC, dried and then baked in a vacuum oven at 80.degree. C. for 2 hr. The duplicate filters were pre-hybridized at 42.degree. C. for 4 hr with 10 ml per filter of DNA hybridization buffer (5.times.SSC, pH 7.0, 4.times.Denhardt's solution (polyvinylpyrrolidone, ficoll and bovin serum albumin, 1x=0.02% of each), 0.1% SDS 50 mM sodium phosphate buffer, pH 7.0 and 100 .mu.g/ml of denatured salmon sperm DNA. <sup>sup</sup>.32 -labeled probes were prepared by kinasing the primer with labeled ATP. The filters were hybridized to 5.times.10<sup>sup</sup>.6 cpm/ml of <sup>sup</sup>.32 P-labeled primer in 1-5 ml per filter of DNA hybridization buffer at 64.degree. C. for 8 hr.

The filters were washed once at room temperature for 10 min in 0.1% SDS. 20 mM sodium phosphate (buffer) and 6.times.SSC; once at 37.degree. C. for 20 min in buffer and 2.times.SSC; once at 50.degree. C. for 20 min in buffer and 2.times.SSC; and finally at 60.degree. C. for 20 min in buffer and 1.times.SSC. The filters were air dried and autoradiographed at -70.degree. C. for 4 hr.

Since the oligonucleotide primer was designed to create a new HindIII restriction site in the mutagenized clones, RF-DNA from a number of the clones which hybridized with the primer were digested with this restriction enzyme. One of the mutagenized clone 4.1 plaques which has a new



HindIII restriction site (M13-AW701) was picked and inoculated into a culture of DG98, ssDNA was prepared from the culture supernatant and dsRF-DNA was prepared from the cell pellet. The correct sequence is confirmed by dideoxy sequencing.

The correctly synthesized strands were isolated and cleaved with PstI and HindIII (partial) or with HindIII alone for ligation into expression vectors.

#### EXAMPLE 24

##### Expression of TNF

For procaryotic expression the coding sequence (along with some 3' untranslated nucleotides) was excised from dsM13-AW701 in two ways:

In the first method, the dsM13-AW701 was digested with PstI and then digested partially with HindIII to obtain the HindIII-PstI TNF coding sequence. (partial HindIII digestion is required because there are several HindIII sites in M13-AW701.) The partial digestion of the DNA fragment can be accomplished by using one-tenth the amount of restriction enzyme required for complete digestion of the DNA. The mixture was incubated at the appropriate temperature for the enzyme and aliquots of the digestion mixture were removed at 10 min intervals for up to 1 hr. The aliquots were then loaded onto a gel and the DNA fragments analyzed. The time point that provided the highest yield of the DNA fragment needed was chosen for a preparative digestion with the restriction enzyme and the appropriate fragment purified from the gel by electroelution.

The PstI/BamHI fragment containing the 3'-non-coding sequence of the TNF gene (see FIG. 2) was purified from pE4 following digestion of the DNA with the enzymes PstI and BamHI.

Together, the HindIII/PstI and PstI/BamHI fragments comprise the coding sequence plus a 600 bp 3' untranslated portion of DNA. The two fragments were ligated into HindIII/BamHI digested host vector pTRP3 as follows:

pTRP3 (ATCC 39946), contains the E. coli trp promoter and ribosome binding site. pTRP3 was digested with HindIII and BamHI, and the vector fragment purified on agarose gel. The isolated fragment was then ligated with the above HindIII/PstI and PstI/BamHI segments in a 3-way ligation, and the mixture used to transform E. coli MM294 to Amp.RTM., giving pAW701.

In a second method. dsM13-AW701 was digested with HindIII and the fragment containing the gene isolated on agarose gel. The isolated fragment was ligated with HindIII cleaved, Bapped pTRP3, and transformed into E. coli MM294 to obtain pAW702.

pFC54.t (ATCC 397B9) or pPLOP (ATCC 39947). containing the P.sub.L promoter and bacillus positive retroregulatory sequence can also be used as host vectors. These vectors are digested with HindIII and BamHI and the large plasmid fragments containing the control sequences purified on agarose gel. The HindIII/PstI and PstI/BamHI portions of the TNF gene, prepared as set forth above, are ligated, in a three way ligation, into the HindIII and BamHI sites of these vectors resulting in plasmids pAW711 and pAW712 respectively. Alternatively, the purified HindIII fragment from mutagenized pE4 is ligated into HindIII cleaved. Bapped pFC54.t or pPLOP to give pAW713 and pAW714, respectively. Plasmid pAW711 was deposited with ATCC on Nov. 8, 1984 and has accession No. 39,918.

pAW701 and pAW702 were transferred into E.coli MM294 and the cultures grown under conditions which suppress the trp promoter. Upon induction by tryptophan depletion, production of TNF was

initiated. In an analogous fashion, pAW711 was constructed and transferred into E.coli MC1000-39531, and the cells were induced by high temperature. After several hours of culturing under induction conditions, the cells were sonicated and the sonicates verified to contain TNF by the L-929 cytotoxicity assay. The results are:

	Plasmid U/ml
701 1.3 .times. 10.sup.4 702 1.3 .times. 10.sup.4 711	
2 .times. 10.sup.5	

Units of TNF activity are as defined below in Example 29.

The vector pB11 isolated from the cDNA library above, contains the SV40 promoter in operable linkage to the TNF coding sequence. All of the 28 positively hybridizing colonies would be expected to contain this linkage, including, specifically pE4 and pB11, and are thus capable of expression in suitable mammalian hosts. Accordingly, pB11 was used to transform COS-7 monkey kidney cells, and the cells cultured under conditions which effect the induction of the SV40 promoter. As the signal sequence is still present in pB11, and functions in mammalian cell systems, the TNF was secreted into the medium. TNF was assayed in the supernatant above the monolayered COS-7 cells by .sup.35 S release from L-929 cells, with results as follows:

	Plasmid .sup.35 S Release (cpm)
B11 22,763 E9 (neg control) 2,739 DNA 2,565	

## EXAMPLE 25

### Preparation of Coding Sequence and Expression Vectors for TNF Muteins

Clone 4.1 prepared by PstI treatment of pE4 as described in Example 23 was subjected to site-specific mutagenesis substantially as described in Example 23, but using as primer 5'-CATGGGTGCTCGGGCTGCCTT-3', which is complementary to the sequence adjacent to the cysteine at position 69, but contains nucleotides complementary to that codon so as to effect a change from TGC to AGC. Mutagenized plaques were identified and confirmed by sequencing as described above. One plaque containing the desired mutation, MB-AW731 was digested with AvaI and PstI, and the fragment ligated into PstI/AvaI digested pAW711. The ligation mixture was transferred into E. coli MC1000-39531 to Amp.RTM. and the transformants screened with the primer probe for the correct sequence. One colony, designated pAW731, was used for expression of the modified sequence. pAW731 was deposited with ATCC Jan. 25, 1985 and has accession No. 53007.

In an analogous manner, pAW741, an expression vector for ser.sub.101 TNF was prepared using the primer CAAGAGCCCCTCTCAGAGGGAG.

## EXAMPLE 26

### Expression of the Coding Sequence for and Activity of TNF Muteins

E. coli MC1000-39531 harboring pAW731 was grown and induced at high temperature in the manner set forth in Example 24. The sonicates from the induced cells were assayed and found to have approximately the same TNF activity per ml as the pAW711 transformants. However, SDS analysis showed that the amount of 17 kD TNF protein in these extracts is about 5x less, showing that the specific activity of Ser.sub.69 TNF is higher than that of the natural or wild-type recombinant TNF

protein.

#### EXAMPLE 27

##### Expression of the Coding Sequence for Recombinant Ser.sub.69 Ser.sub.101 Human TNF Mutein

Plasmid pAW731 (ser69) is digested with HindIII and HincII, and the small HindIII-HincII fragment containing the ser69 mutation is purified on agarose gel. Similarly, plasmid pAW732 (ser101) is digested with the enzymes HincII and BamHI, and the HincII-BamHI fragment containing the ser.sub.101 mutation is purified. The previously purified HindIII-BamHI vector fragment from pFC54.t is then ligated with the HindIII-HincII (ser69) fragment and the HincII-BamHI (ser101) fragment to generate the ser69ser101 TNF clone, pAW735. This dimutein, ser69ser101 TNF, is less likely to dimerize on purification due to the absence of any free cysteines.

#### EXAMPLE 28

##### Cysteine Residues in Native or Wild-Type Human TNF are not Needed for Biological Activity

Purified (95%) unaltered protein was reduced and alkylated by treating with DTT and iodoacetate according the protocol set forth below. While the untreated protein had an activity in U/ml of 2.6.times.10.sup.4, reduced or reduced alkylated protein had activities of 4.4-4.8.times.10.sup.4 U/ml:

	Treatment	Activity
	No DTT	2.6 .times. 10.sup.4
0.1 mM DTT	3.3 .times. 10.sup.4	
1 mM DTT	4.8 .times. 10.sup.4	
2 mM DTT	3.9 .times. 10.sup.4	
10 mM DTT	1.2 .times. 10.sup.4	
20 mM DTT	1.7 .times. 10.sup.4	
buffer + 2.4 mM IAA	1.5 .times. 10.sup.4	
1 mM DTT + 2.4 mM IAA	4.4 .times. 10.sup.4	

The above data demonstrates that the cysteines in the wild-type recombinant human TNF are not required for biological activity, thus, one or both of the cysteines may be deleted or substituted with another amino acid as provided within the scope of the invention.

#### EXAMPLE 29

##### Cytotoxic Assay procedure for TNF

The L-929 assay system is an improved convenient in vitro assay which permits rapid measurement of TNF activity. Its degree of correlation with the in vivo tumor necrosis assay of Carswell (supra) is, at present, unknown; however, as it utilizes murine tumor cells specifically, the correlation is expected to be high. The protein designated lymphotoxin in EPO publication No. 0100641 also gives activity in this assay. The assay is similar in concept to that disclosed in U.S. Pat. No. 4,457,916 which used murine L-M cells and methylene blue staining. However, the L-929 assay has been shown herein to correlate (for HL-60-derived TNF) with human tumor cell line cytotoxicity.

In the L-929 assay system, L-929 cells are prepared overnight as monolayers in microtiter plates. The test samples are diluted 2-fold across the plate UV irradiated, and then added onto the prepared cell monolayers. The culture media in the wells are then brought to 1 .mu.g/ml actinomycin D. The plates are allowed to incubate 18 hr at 37.degree. C. and the plates are scored visually under the microscope. Each well is given a 25, 50, 75 or 100% mark signifying the extent of cell death in the well. One unit of TNF activity is defined as the reciprocal of the dilution at which 50% killing occurs.

In addition a more sensitive version of this assay was developed that monitors the release of <sup>35</sup>S labeled peptides from prelabeled cells, when treated with the test sample and actinomycin D. This version of the assay can be used to quantitate potency, e.g. to evaluate the relative potency of oocyte translated material. Briefly, actively growing L-929 cultures are labeled with <sup>35</sup>S methionine (200  $\mu$ Ci/ml) for 3 hr in methionine-free media supplemented with 2% dialyzed fetal calf serum. The cells are then washed and plated into 96 well plates incubated overnight, and treated the next day with 2-fold dilutions of test samples and 1  $\mu$ g/ml actinomycin D. The cultures were then incubated at 37.degree. C. for 18 hr. 100  $\mu$ l supernatant aliquots from each well were then transferred onto another 96 well plate, acid (TCA) precipitated, and harvested onto glass fiber filters. The filters were washed with 95% ethanol, dried and counted. An NP.sub.40 detergent control is included in every assay to measure maximum release of radioactivity from the cells. The percent <sup>35</sup>S release is then calculated by the ratio of the difference in count between the treated cells and untreated controls divided by the difference between NP.sub.40 treated cells and untreated controls, i.e., by the ratio: ##EQU1## Higher TNF potency results in higher values of this ratio.

The TNF muteins of the invention are conveniently formulated into suitable therapeutic formulations which will typically includes a therapeutic effective amount of the mutein and a suitable physiologically acceptable carrier as described above with respect to the IL-2 muteins. Alternatively, other cytotoxic, antiviral or anti-cancer agents may be used in combination with the TNF muteins of the invention such as gamma interferon.

On Oct. 15, 1984, Applicants have deposited with the American Type Culture Collection, Rockville, Md., U.S.A. (ATCC) the plasmid pE4. described hererin ATCC accession No. 39,894. On Nov. 8, 1984, pAW711 was deposited and given ATCC accession no. 39,918. On Jan. 25, 1985, pAW731 was deposited and given ATCC accession No. 53,007. These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of patent procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent US patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Modifications of the above described modes for carrying out the invention that are obvious to those of skill in the fields of genetic engineering, protein chemistry, medicine, and related fields are intended to be within the scope of the following claims.

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